













# THE JOURNAL

OF

# EXPERIMENTAL ZOÖLOGY

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VOLUME 29

1919

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY  
PHILADELPHIA, PA.





# CONTENTS

## No. 1. AUGUST

E. R. HOSKINS AND M. M. HOSKINS. Growth and development of Amphibia as affected by thyroidectomy. Nine plates (one hundred and nine figures).....	1
HELEN DEAN KING. Studies on inbreeding. IV. A further study of the effects of inbreeding on the growth and variability in the body weight of the albino rat. Eight charts.....	71
HARLEY N. GOULD. Studies on sex in the hermaphrodite mollusc <i>Crepidula plana</i> . III. Transference of the male-producing stimulus through seawater. One text figure.....	113

## No. 2. OCTOBER

GARY N. CALKINS. <i>Uroleptus mobilis engelm.</i> II. Renewal of vitality through conjugation. One chart and one figure.....	121
LESLIE B. AREY AND W. J. CROZIER. The sensory responses of <i>Chiton</i> . Fourteen figures.....	157
W. J. CROZIER AND LESLIE B. AREY. Sensory reactions of <i>Chromodoris zebra</i> . Eight figures.....	261
MATSUZO TAKENOUCHI. Studies on the reputed endocrine function of the thymus gland (albino rat). Two charts.....	311

## No. 3. NOVEMBER

DWIGHT E. MINNICH. The photic reactions of the honey-bee, <i>Apis mellifera</i> L. Seventeen figures.....	343
ROBERT W. HEGNER. The effects of environmental factors upon the heritable characteristics of <i>Arcella dentata</i> and <i>A. polypora</i> . Seven figures....	427
M. E. COLLETT. The toxicity of acids to ciliate Infusoria. Six graphs....	443
J. A. DAWSON. An experimental study of an amiconucleate <i>Oxytricha</i> . I. Study of the normal animal, with an account of cannibalism. Twenty figures.....	473





# GROWTH AND DEVELOPMENT OF AMPHIBIA AS AFFECTED BY THYROIDECTOMY

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NINE PLATES (ONE HUNDRED AND NINE FIGURES)

## CONTENTS

Introduction.....	2
Technique.....	3
Results.....	4
<i>Amblystoma punctatum</i> .....	4
Growth.....	4
Microscopic changes.....	4
<i>Rana sylvatica</i> .....	5
Growth of body.....	6
Length and volume.....	13
Regeneration.....	15
Forced metamorphosis.....	16
Discussion of growth and metamorphosis.....	17
Organs, and method of comparison (discussion follows description of each organ).....	19
Brain.....	21
Eyeballs.....	22
Heart.....	22
Liver.....	22
Hypophysis.....	23
Thymus.....	29
Epithelioid (parathyroid) bodies.....	32
Thyroid.....	33
Spleen.....	36
Kidneys.....	36
Gills and lungs.....	37
Intestines.....	38
Adrenals.....	38
Skin.....	38
Ovaries.....	39
Testes.....	43
Summary and conclusions.....	46
Literature cited.....	49

## INTRODUCTION

One of the experimental methods most frequently used in the study of the thyroid gland is that of extirpation. Mammals have usually been employed in this study and have usually proved unsatisfactory for definite results, especially where data on the relation of the thyroid to growth processes were sought for. In most mammals complete thyroidectomy is followed shortly by death. Moreover, in such animals the gland cannot be removed until after it has functioned for a time. Still further, growth processes are relatively rapid in the higher animals. We thought that if use were made of a lower form wherein growth processes are relatively slow and wherein a larval existence would permit extirpation of the thyroid before it could possibly have become functional, a new approach would be opened to the problem of the relation of the thyroid to growth and to life processes in general. With this in mind, we selected amphibia for our material. An added interest lay in the fact that Guder-natsch ('12, '14) had clearly shown that the opposite sort of experiment, i.e., feeding thyroid substance to larval frogs, alters remarkably their growth processes and hastens metamorphosis. The forms selected were *Rana sylvatica* and *Amblystoma punctatum*, which are very abundant about New Haven.

The first operations were performed by one of us in April, 1916, and in the spring of 1917 and 1918 the experiments were repeated. We learned in November, 1916, that Doctor Allen, of the University of Kansas, had performed similar experiments with *R. pipiens* at about the time when our first were made. Doctor Allen's and our own work were done independently of each other, neither of us at the time knowing of the other's experiments. We wish to thank Prof. R. G. Harrison for the kind interest he has taken in the work and for his very helpful suggestions. Abstracts of different portions of this paper have already been published (Hoskins and Morris, '17; Hoskins and Hoskins, '18).



## TECHNIQUE

The best larval (or embryonic) stage for a study of this kind is that just preceding the beginning of the circulation of the blood, as at this time hemorrhage is avoided and the chance of regeneration of the removed tissue is less than if younger stages are used. The *R. sylvatica* larvae at the time of operation were 5 to 8 mm. in length and the *Amblystoma* larvae were 10 mm. in length. The animals were anesthetized and were operated upon in chlorotone as recommended by Harrison ('04). The solution used was 0.02 per cent chlorotone in 0.3 per cent sodium chloride.

A layer of paraffin blackened with carbon was put into a Syracuse watch-glass and in it were made six grooves, each sufficiently wide to admit an embryo lying ventral side up. After the thyroid gland was removed the embryo was left in position for about an hour or until five other operations had been performed, the first then being removed to make room for the seventh. By that time the wound had sufficiently healed to permit handling with a pipette. Asepsis was found to be unnecessary.

The operations were performed under a binocular microscope. A transverse incision was made with fine scissors through the ectoderm posterior to the oral plate, the flap of ectoderm covering the thyroid anlage was pulled caudally, the mesenchyma pushed laterally or removed, and the exposed anlage of the thyroid was removed together with that portion of the ventral wall of the pharynx surrounding its proximal margin. The thyroid being opaque is readily distinguished from the translucent mesenchyma. Care must be taken to avoid injury to the pericardium and heart. Usually the wound appeared to be healed in two or three hours. After the operation (one to two hours) the salt solution was gradually diluted with tap-water. It was found that the embryos were harmed by being left too long in the saline. Many *sylvatica* larvae were kept together in the same aquarium, but the *Amblystoma* larvae were isolated to prevent their eating one another's gills.

## RESULTS

*Amblystoma*—1916

*Growth.* There were no constant external changes evident among the thyroidectomized *Amblystoma* larvae in the three months after the operation during which the animals were under observation. At the end of this time all those surviving were killed for microscopic examination. The gills and legs developed normally. The average length of the seven surviving controls at this time was less than that of the seven experimental larvae, but the number of specimens was too small for final conclusions with regard to growth rate, especially since the largest of the fourteen was one from which the thyroid had been removed. The largest thyroidless larva was 30 mm. long and the largest control 24 mm. One probable source of variation in growth was the food supply, as the animals were isolated and may very likely have had unequal amounts of food.

*Microscopic changes.* Four days after the operation the animals had attained the length of 12.5 mm. Sections of control animals show that the thyroid at this time is long and narrow, lying ventral to the pharynx and anterior to the heart. In the animals from which the thyroid had been removed the floor of the pharynx healed without regeneration of the gland. The hypophysis was not structurally different from that of the controls in this stage.

Three months after the operation there were still no differences to be observed between sections of the control and experimental *Amblystoma* larvae, except of course absence of the thyroid from the latter. The cartilages and muscles ventral to the pharynx developed normally despite the removal of mesoderm in this region. The thyroid glands, which were lacking in all the experimental animals were well developed in the controls at this time (figs. 1, 2, and 5). The vesicles are found scattered along the ventral aortae. Colloid is abundant, although the cells have very little cytoplasm, the wall of the follicle being very thin in most cases. The gland is about 0.4 mm. in length and of variable shape. Since none of the thirteen experimental

larvae which were sectioned showed any thyroid tissue, it is probable that the operation was successful in most if not all cases.

The hypophysis (figs. 3 and 4) three months after the operation exhibited no peculiarities of structure in the thyroidless animals that could be attributed to the operation. Micrometric determinations averaged practically the same as in the control animals when allowance was made for the size of the animals in each case. The hypophysis shown in figure 3 (control) is smaller than that in figure 4 (operated), but the entire animal was also smaller. The cells of the hypophysis are arranged in clusters in both larvae, but there is no other evidence of functional differentiation.

*R. sylvatica*—1916-1918

A comparison of figures 6 and 7 of *R. sylvatica* of 6.5 mm. in length shows the longitudinal dimension of the area of entoderm removed in the operation. This included not only the entire anlage of the thyroid gland, but also a considerable portion of the floor of the pharynx and a part of the surrounding mesenchyma. The anlage in this stage is comparatively large. It extends caudally and ventrally to the pericardium, but with care it may be removed without injury to the latter. Blood-vessels removed during the operation usually regenerated. The hypophysis (fig. 6) is already ventral to the infundibulum. It does not appear in figure 7 because of the orientation of the specimen for sectioning.

Figures 8 and 9 of larvae four days after the operation show that the floor of the pharynx and mouth have been completely regenerated. The thyroid which is well developed in the control animal (fig. 9) is entirely missing from the operated larva (fig. 8X). The hypophysis is well formed in both specimens and shows no difference in size or structure at this time, although in older thyroidless tadpoles it is found to have undergone hypertrophy. No other changes in the organs are noticeable until the time of metamorphosis.

In the larva represented in figure 8 and in many other specimens the removal of mesoderm with the thyroid anlage had no effect on the development of the muscles and cartilages of this



region. In some cases, however, some of the muscles failed to develop properly, and this has an important effect on the growth of the animal. This will be discussed later.

Among the frog larvae from which the thyroid had been removed a few animals developed abnormal external gills, the shape of which was variable. Some of these were low and flattened, some long and slender, but irregular, and with a variable number of filaments. In other specimens the filaments were club-shaped being enlarged at the distal ends. The circulation through these abnormal gills was often sluggish. One experimental frog larva did not develop any external gills, although it lived and grew otherwise apparently normally through the period in which external gills normally persist. These branchial malformations were considered to be the result of vascular injury inflicted during the operation. Ekman ('13) states that the blood-vessels are necessary for the later development of the external gills.

The twisting of the tail which is common among frog larvae kept in aquaria occurred in our 1917 specimens which were not under optimum conditions. It was also noted in the regeneration experiment described below. It did not occur at all in the rapidly growing 1918 larvae. The abnormality is not due to hardness of water, but to food conditions, for the same kind of water was used in both years.

*Growth.* In our 1916 experiments it appeared that the average growth rate was less in the operated animals than in the controls, and it was so reported (Hoskins and Morris, '17). The reason for this difference in the average rate of growth we later discovered to be due to two chief causes, and the real difference is just the opposite from what it appeared to be. In some cases the operation injures the developing lower jaw and its muscles, especially the submaxillary; and these animals have difficulty in eating, and suffer from partial or complete inanition. In other animals the thyroid is not completely removed, and regenerates, and these animals grow at about the same rate as the controls. The experimental larvae may be divided into three groups: those with defective jaws or muscles which grow

less rapidly than the animals of the control group; those with regenerated thyroids which grow at about the same rate as the controls; and those which have normal jaws and muscles, but are devoid of thyroid, and which grow more rapidly than the normal animals.

The growth rate of frog larvae kept in aquaria varies greatly, and the most important factor in this is the kind and quantity of food; but the size of the aquarium is very important, because it is easier to keep large aquaria balanced than small aquaria. The influence of the food and size of the aquarium is shown very clearly by a comparison of the growth of our 1917 and 1918 animals.

In 1917 fourteen series of extirpations were performed with about 700 larvae. These were kept in small aquaria and fed from April to the beginning of June upon a plant obtained from a local aquarium supply dealer, and to this diet were added occasionally cracker crumbs and bits of liver. In June the diet was changed to spirogyra, and the animals were transferred to larger aquaria. The growth rate rapidly increased when this change was made. The average time of metamorphosis of the control animals did not arrive until the first week in August. Under optimum laboratory conditions *R. sylvatica* undergoes metamorphosis in June or July.

In 1918 the animals were exposed to the sun for an hour or two each day. In each aquarium were placed small plants and a layer of mud and old oak leaves from a pond of stagnant water. The diet used in 1918 was composed principally of algae found floating on the surface of a stagnant pond. Taken with the algae were large numbers of small crustaceans which the tadpoles may have eaten after the crustaceans died in the aquaria. Only small amounts of the algae should be put into the aquarium at a time, because if added in large amounts it will die and poison the tadpoles. In our 1918 experiments the animals grew much more rapidly than did those of the previous years. Whereas in 1916 and 1917 the larvae required a minimum of three months to reach their maximum size and begin metamorphosis, the minimum time required by the 1918 larvae for this was only

one month—a time much less than that usually required for *R. sylvatica* kept in the laboratory. In the former series the maximum time required for metamorphosis to begin was four months, while in the latter series the corresponding time was only seven weeks.

The growth of our experimental and control animals seems to have been different from Allen's ('18), who states that he observed no difference between the controls and thyroidless larvae until the time of metamorphosis. This point we studied very carefully by taking hundreds of measurements of the growing animals. As shown in table 1, we found that in every one of the experiments except one small series (5/5) in which the thyroidless larvae all had defective jaws or muscles, the largest thyroidless larvae grew more rapidly than the largest controls. The same was true also of the average after elimination of those suffering from inanition. For a few days after the operation the larvae grew more slowly than the controls, but passed them at an average size of 18 mm. (range 12 to 23 mm.). At this time there were alive 260 operated and 218 control larvae, so the fact of more rapid growth of the former seems well established. The 1918 series were not measured at this stage. This difference in growth rate could not be accidental or due to the food supply, because it was constant in every series from 1916 to 1918 larvae, the latter of which were especially well fed. By the time of metamorphosis of the controls the largest corresponding thyroidless larvae had attained the length of 58 mm., and in every series those thyroidless larvae without defects were all larger than the controls when the latter had reached their maximum size. One control larva became 53 mm. long before metamorphosis and one reached the length of 50 mm., but with these two exceptions none of the controls ever grew longer than 48 mm. before metamorphosis, the smallest being only 40 mm. long, and the average 46 mm. before they became transformed into frogs.

In general, the 1917 larvae, which grew more slowly than the 1918 animals, became larger than the latter before metamorphosis, but the largest in 1918 were larger than the average of



TABLE 1

Showing that the removal of the thyroid from small frog larvae hastens their growth.  
 Thyroidless larvae which do not have defects of the lower jaw or  
 its muscles grow more rapidly than the controls  
*R. sylvatica*

DATE OF OPERATION	LENGTH WHEN OP. EXCEED CON. MM. AVER.		NUMBER WHEN OP. EXCEED CON.		NUMBER WHEN CON. MET.		NUMBER OF OP. NOT MET.	LENGTH AT TIME WHEN CON. MET. MM.		MAX. LENGTH OF OP. MM.
	Op.	Con.	Op.	Con.	Op.	Con.		Op.	Con.	
1916	nx.		nx.		1	1	1			
1917										
April 17.....	17.0	15.0	8	9	0	2			53 <sup>1</sup>	
April 9.....	18.5	18.0	10	20						
April 10.....	nx.		nx.		2	0	2	50		Killed at 56
April 11.....	12.0	11.5	12	12	1	1		42	40	
April 12.....	15.5	14.5	50	18	9	0	1	47		Killed at 62
April 14.....	17.5	17.0	37	27	2	0	2	58		68
April 15.....	19.5	19.0	12	8	1	1	1	55	45	70
April 27.....	21.0	19.0	8	21						
April 28.....	23.0	20.0	15	15	11	0	9	59		68
April 30.....	20.5	19.0	34	24	3	4	2	49	48	67
May 1.....	19.0	17.0	40	30	7	3	7	54	47	72
May 3.....	18.7	16.5	25	22	5	3	5	51	47	68
May 5.....	17.5	18.0	8	6						
May 6.....	29.5	29.0	1	6	1	6	1	53	50	72
1918										
April 14.....	30.0	23.0	9	55	9	55	7	55	48	Killed at 65
April 22.....	nx.		nx.		11	30	11	56	46	Killed at 56
May 2.....	38.0	32.0	29	13	28	13	26	48	44	Killed at 48
Aver. max. { 1916-17....	18.3	17.2	260	218	43	21	31			
or total { 1916-18....					91	119	75	58	53 <sup>1</sup>	72

N. B.—The 1918 larvae were not measured at the 18 to 20-mm. stage. The 5/5 group of thyroidless larvae had defective jaws. Abbreviations: *Aver.*, average; *Con.*, control larvae; *Op.*, thyroidless larvae; *Nx.*, not measured; *Max.*, maximum; *Met.*, metamorphosis or metamorphosed.

<sup>1</sup> Only one of all the control larvae studied became over 50 mm. in length.

1917. The frogs which formed from the larger larvae were larger than those formed from smaller larvae. In *Rana sylvatica* there is thus a great variation in the size of the newly formed frogs. As shown in table 2, the body length of these normal frogs of the same stage of development varies more than 25 per cent and their volume nearly 100 per cent.

The mortality in 1917 was very great, but was about the same in both the thyroidless and control animals. However, 42 of the former and 20 of the latter reached the period of normal metamorphosis. Of these (table 1) the controls all metamorphosed normally. Of the operated animals, 12 metamorphosed and 30 failed to do so. By autopsy and sectioning it was later ascertained that every one of the 30 was devoid of thyroid, and in every one of the 12 the gland had regenerated after the operation. In the 1918 work, out of 47 thyroidless animals which reached the period of normal metamorphosis, 44 failed to metamorphose and in only three experimental larvae did the thyroid regenerate permitting metamorphosis. In the three seasons a total of 91 experimental larvae were reared to or beyond the time of normal metamorphosis, and of these 75 remained in the larval condition. In addition to these, many which were killed in the early stages for purpose of study would doubtless have survived long enough to be added to the 75. From these experiments and those of Allen ('18) we are justified in the conclusion that removal of the thyroid from young frog larvae will delay if not entirely prevent metamorphosis under ordinary laboratory conditions. Most of the 75 thyroidless larvae just referred to were killed after it became evident that they would not develop into frogs, but a few were kept alive. Of these, six survived the winter and the second season of normal metamorphosis without becoming frogs. It is more or less common knowledge that if *Amblystoma* larvae kept in aquaria are not properly fed, they may fail to undergo metamorphosis their first season, but do metamorphose the second season. The same thing is true also in nature. Dr. T. G. Lee, of the University of Minnesota, has told us that he has collected in the early spring *Amblystoma* larvae which should have metamorphosed the previous season.

Normal *Rana sylvatica* larvae kept in aquaria and well fed metamorphose normally, but with our thyroidless frog larvae the second season was passed without their metamorphosing.

Concerning the growth of the thyroidless larvae, it may be noted from tables 1 and 2 and figures 10 to 19 that they ultimately became much larger than the control larvae. As stated above, the thyroidless animals grow more rapidly than the controls, but the difference is not very great until near the time of metamorphosis. When the controls at this period have nearly reached their maximum length, their growth seems to slacken, and their length does not change for a short period of time before it starts to decrease during metamorphosis. It is during this time, as was noted also by Allen ('18), that the experimental animals gain very much in size over the controls. In some of our 1918 series measurements of volume of the animals were made at this time and in some cases (table 2) the thyroidless larvae were more than twice as large in volume as the control animals of the same age. The volume was determined by the amount of liquid displaced by an animal. After the metamorphosis of the controls (1917) the growth rate of the experimental larvae decreased greatly and growth nearly ceased during the winter, but the larvae grew more rapidly during the second spring and summer. For example, the animal shown in figure 19 became 55 mm. in length by the time the controls had metamorphosed in early August, 1917. It was 62 mm. in length on September 2 and 66 mm. on September 27 when next measured. On October 19 the length had not increased. The animal began growing again in May, 1918, but it increased its length by only 5 mm. by June 25, and on July 5 had reached its maximum length of 72 mm. with a volume of 2.38 cc., or nearly three times that of the normal larva at the time of metamorphosis. In this growth after the first summer the body length (nose-anus) increased 2 mm. and the tail 8 mm. the animal thereby becoming relatively long-tailed. Its hind legs increased after the first season about one-half of a millimeter in length, reaching 5 mm. In these miniature legs the normal segments and digits are slightly differentiated.



One 1917 operated larva deserves special mention. Its thyroid regenerated and then hypertrophied. The animal metamorphosed about a month before the controls and after metamorphosis it was less than one-third the normal size (fig. 20). The length of the controls at this time was 40 mm. (fig. 21). An attempt was made to duplicate this experiment in 1918 by cutting to pieces the thyroid anlage and leaving the pieces in the animal. In other specimens transplantation of the thyroid was performed, but in neither group did these experimental larvae metamorphose earlier than the controls, although a few were smaller than normal. In three 1918 specimens from which the thyroid had been removed, the gland regenerated and the animals metamorphosed at a size about one-half that of the average control larva, but not at an earlier date.

Figures 10 to 19 show the changes in body form exhibited by the control and thyroidless animals. Figures 12 and 13 show a normal tadpole in the process of metamorphosis. The hind legs are well developed and the skeleton of the body has begun to acquire the adult shape. In figure 12 it may be noted that the head is flattening and narrowing. The larva shown in figures 14 and 15 is a thyroidless specimen of the same age as that of figures 12 and 13. Its total length is greater than that of the control larva, the hind legs are very small and the body (fig. 14) is more nearly cylindrical than that of the control (fig. 12). The eyes are more laterally placed in the control larva and, although its head is narrower than that of the other (fig. 15), the eyes are further apart. The head of the control specimen is slightly more pointed than that of the thyroidless larva and is more like that of the frog (fig. 17). The experimental animal retains this larval form for some time, but it slowly undergoes changes in shape which tend toward those of the controls. Normally, during the period of metamorphosis, the form of the animal changes as follows: The body decreases very slightly in length; the anal canal shortens 2 to 4 mm. (compare figs. 12 and 16); the transverse and horizontal diameters decrease nearly one-half; the tail atrophies; the dorsal and ventral sides flatten; the eyes come actually closer together, but are relatively farther

apart; the rima oris increases in size and changes in shape; the nostrils become relatively more lateral in position and larger, and the legs increase from one-half to two-thirds (table 2).

The thyroidless larvae after the season of normal metamorphosis still resemble young larvae in general shape, but they

TABLE 2

*Length and volume of larvae and frogs. Showing the variations in the length and volume of living and fixed larvae. The animals were fixed in picro-formo-acetic and preserved in alcohol. Note the small size of the frogs, which are only one-third to one-half the volume of the larvae from which they are transformed. Variations in shrinkage occur during fixation.*

CONTROL LARVAE IN ALCOHOL			THYROIDLESS LARVAE						FROGS IN ALCOHOL		
			Alive			In alcohol					
			Length		Volume	Length		Volume	Length		Volume
N-A.	Tot.	N-A	Tot.	N-A.		Tot.	N-A.		Tot.	N-A.	
mm.	mm.	cc.	mm.	mm.	cc.	mm.	mm.	cc.	mm.	mm.	cc.
20.0	45.0	1.10	25	72.0	2.38	24.5	68.5	1.92	16.0	20.0	0.42
19.0	46.5	0.81	25	65.5	2.40	23.0	60.0	1.80	16.0	18.0	0.40
19.0	41.5	0.76	25	65.0	2.45	20.0	54.0	2.19	15.0	19.0	0.35
18.5	46.0	0.95	25	65.0	2.41	22.5	58.0	1.95	15.0	17.0	0.39
18.5	46.5	0.86	25	63.0	2.37	23.0	60.0	1.80	14.5	15.0	0.31
18.5	46.5	0.79	24	64.0	1.98	22.0	57.0	1.78	14.0	15.0	0.28
18.0	46.0	0.99	22	63.0	1.88	21.0	56.0	1.58	14.0	15.0	0.23
18.0	42.0	0.78	20	54.0	1.19	17.0	48.5	0.98	13.5	16.0	0.30
18.0	41.0	0.68	18	46.0	0.85	17.0	42.5	0.75	13.0	19.0	0.26
17.0	42.5	0.74	16	39.5	0.60	15.0	36.5	0.50	13.0	13.5	0.22
17.0	39.0	0.55	14	38.0	0.51	13.7	34.5	0.41	12.5	14.0	0.22
16.5	40.0	0.73	13	32.5	0.38	12.0	30.0	0.32			
16.0	39.5	0.52									
Averages 18	43.2	0.79	21	55.6	1.57	17.6	50.5	1.33	14.2	16.5	3.07

N-A., Nose-anus length; Tot., total; Vol., volume.

have undergone a few changes (figs. 18 and 19). The tail has become relatively long, the eyes are wider apart, the dorsum of the head is slightly flattened, and the legs have increased very slightly in length. During the winter and the second spring and summer the thyroidless larvae (figs. 10 and 11) make a still nearer approach in form to that of the frog. The legs

increase very slightly in length, the head and back become very flat on account of skeletal changes, and the eyes are relatively more lateral in position than in normal larvae. Other changes noticeable are a relative increase in the length of the tail and a blunting of the anterior end of the head. Allen ('18) states that the head is relatively long, but this is not the case in our specimens. In his comparison Allen ('18, fig. 3) failed to superimpose the centers of the heads of the larvae. At the end of the period of metamorphosis the thyroidless larvae are from three to five times as large in volume as the frogs of the same age and some become ultimately more than six times as large as the average frog after metamorphosis and nearly three times as large as the average control larva before metamorphosis (table 2).

Table 2 shows the variation in the volume (and hence weight) of amphibians as compared on a basis of length both in the living and fixed condition. These animals were fixed in Bouin's fluid and preserved in 70 per cent alcohol. The data here were selected from a very large number of such measurements and published in order to demonstrate a source of serious error in attempts to compare relative volumes of organs on a basis either of length or volume of the animal, as well as to show the relative sizes of larvae and frogs. The volume of fixed specimens varies greatly on account of variations in the amount of shrinkage during fixation, and similar variations in the size of fixed organs must also occur.

The volume (or weight) of a living larva of a given length is only fairly constant and varies not only with the amount of foreign substances within the intestine, but also on account of variations in shape produced by changes in the tail-body relationship. The intestinal contents cannot be removed without destroying the gut, and this would prevent the measurement of the volume (or weight) of the entire animal. In addition to loss of water, a considerable portion of the natural decrease in size suffered during transformation from the tadpole to the frog is due to discharge of intestinal contents, and the exact amount of this is difficult to determine. This loss of fecal matter in-



creases the difference between the relative volume of larvae and frogs. The shortening of the tail and anal canal during metamorphosis prevents comparison of the larvae and frogs on a basis of the body length (figs. 12 and 16). Given a frog and larva of the same body length (i.e., nose-anus) the latter is two to three times the volume or weight of the former (table 2). If the body length be considered as the distance between the tip of the head and the posterior limit of the body cavity, a larva is more than three times the size of a frog of the same body length.

In table 2 the measurements of living larvae are given only in the case of the thyroidless animals, as the variation in the controls is similar. The increase in the volume of the control larvae and frogs on account of growth of the legs is offset by a slight increase in the relative length of the tail in the thyroidless animals.

*Regeneration.* On April 13, 1918, 12 mm. of the tail and the entire hind legs were removed from a large thyroidless larva from the 1917 series. After fourteen days the tail had increased in length 11 mm. and the body 2 mm. One hind leg had not grown at all, but the other had grown 2 mm. and regenerated a foot that was about half the size of the one removed. The part of the tail that regenerated contained less muscle than normal but was otherwise like the part removed. On April 27 an additional 13 mm. of the tail and the regenerated leg and foot were removed. The tail increased again 14 mm. in length in twenty-eight days. The leg did not again regenerate. The body again increased 2 mm. in length. On June 24 an additional 12 mm. of the tail was removed and it regenerated 7.5 mm. in twenty-three days. During this period a twist developed in the tail near the body. On July 17 an additional 7.5 mm. of the tail was removed. In five days, 2 mm. of the tail had regenerated when the animal died. During the three months of this experiment none of the other thyroidless larvae had increased more than 6 mm. in total length, 2 mm. of which growth was in body length, while the experimental animal had regenerated 34.5 mm. of tail and its body grew 4 mm. Had it not died, the

larva might have grown still more. Only one hind leg showed any sign of regeneration, and it grew to only half the size of the one removed. When this regenerated leg was removed the animal was unable to regenerate it again.

The steadily increasing amount of time required to regenerate the removed caudal tissues and the failure in regeneration of removed legs indicates that, although thyroidless larvae are able to grow and regenerate tissues more than a year after the extirpation of the thyroid, the larva will not continue to regenerate removed tissues indefinitely or at the same rate as the first regeneration it produces. Allen ('18) has noted that young thyroidless larvae are able to regenerate removed parts of the body. Zeleny ('09) found that in normal Salamander larvae repeated amputation of the tail causes an increase in the rate of regeneration. This is influenced, however, by additional injuries to the body, such as removal of one or both hind legs. If the additional injuries are severe, a decrease in the rate of regeneration is to be observed.

*Forced metamorphosis.* An attempt was made to force a thyroidless larvae into metamorphosis. The total length of the animal was 59 mm.; the nose-anus length, 24 mm.; the length from the nose to the end of the body cavity, 20 mm.; that of the hind legs, 4.5 mm., and the volume was 1.90 cc. This larva was placed in a moist chamber on a bed of wet spirogyra, where it lived for two days. It is probable that with care animals might be kept alive in such conditions much longer. This specimen breathed at irregular intervals. In the forty-eight hours during which it lived in the moist chamber, its tail shrank 24 per cent, its anal canal about 50 per cent in volume (due mostly to discharge of contents), and its volume decreased 18 per cent. The gut shortened little, if any, but was strongly contracted. The lungs were filled with air. The shrinkage was not due to inanition, for if a larva similar to the one described is kept in water without food it will maintain its volume for a much longer period than that given to the experiment. Moreover, the shrinkage which finally results from inanition is uniform, while in this experiment the tail shrank first of all, and very rapidly, as it does

in normal metamorphosis. A part of the shrinkage noted in this experiment was due to loss of water from the tissues, as happens to some extent during normal metamorphosis.

*Discussion.* From the results given in the numerous papers dealing with thyroidectomy in higher animals it was to be expected that definite and striking results would be obtained by thyroidectomy in amphibia. Rogowitsch ('89) found that this operation in mammals causes hypertrophy of the hypophysis and Stieda ('90) noted an increase in the number of 'chief' cells of this gland. Herring ('08) stated that thyroid extirpation in mammals causes increased activity of the pars intermedia of the hypophysis, bringing about colloid formation. He reported also a proliferation of the cells in the posterior lobe. Hofmeister ('94) stated that this operation in mammals produces cachexia, resulting in abnormal growth, especially of the bones. Ceni ('05) stated that thyroidectomy in fowls causes interference with egg production. Gudernatsch ('12, '14, '17) and several others have shown that feeding thyroid to larval frogs hastens the time of metamorphosis and checks general growth, hence results somewhat opposite to those were to be expected in our experiments. The effects produced by feeding thyroid to young amphibia are probably not due to any specific metamorphosing function of the thyroid, but rather to a perversion of metabolism, since they have been produced with thyroid substance of mammals which do not undergo metamorphosis as do the amphibia used in the experiments. Feeding thyroid to mammals increases metabolism and causes hypertrophy of various organs, including some of the ductless glands (Hoskins, '16). It was also to be expected that the operation we performed might affect the hypophysis, since Smith ('16, '17) noted that after hypophysectomy in young frogs embryos the thyroid failed to develop normally, and Allen ('17) obtained somewhat similar results.

The earliest apparent and most extensive differences between the thyroidless and control frog larvae are in the skeletal elements. As Terry ('18) has well described, calcification and ossification (especially the latter) progress less rapidly in the thyroidless larvae than in the controls, and finally these processes



practically cease before the frog skeleton is laid down and before the other parts of the larval body have ceased growing. It is probable, as in mammals, that thyroid deficiency interferes with calcium metabolism and thus causes the skeletal abnormalities noted here. The failure of the legs to grow beyond mere buds may be due to the failure of the skeletal part to push out into these buds.

The beginnings of metamorphosis are to be observed in the skeleton, which changes to accommodate the body of the animal to its future terrestrial existence. Metamorphosis consists of a series of changes which occur in sequence, and when the first part of the process is prevented (i.e., skeletal change) through faulty metabolism (probably calcium) other steps in the sequence are prevented. It is to be noted that the lungs develop and function before metamorphosis begins, and have nothing to do with this phenomenon. As soon as normal larvae are well started in metamorphosis they may be removed from the water, and the process will continue to its completion.

It is of course possible that thyroid secretion is directly necessary for the atrophy of tissues which occurs in metamorphosis, but the experiment in forced metamorphosis described above showed that a certain amount of atrophy will take place in a thyroidless larva kept in a moist chamber.

The hypertrophy of the hypophysis which occurs after thyroidectomy may have something to do with the failure of metamorphosis, but it is more likely that the condition of this gland is responsible for the rapid growth rate of thyroidless larvae.

There is no apparent reason for the fact that the size at which frog larvae go into metamorphosis is not constant. Animals from the same egg mass kept in the same aquarium and hence under similar food and temperature conditions may vary considerably in the size attained before metamorphosis and in the time required for it. As noted above, the 1917 (slowly growing) larvae averaged larger than the 1918 (rapidly growing) larvae, but the food conditions are not the only determining factor, because the largest 1918 larvae were larger than the smaller 1917 larvae. It may be that about the time the larvae reach their

maximum length their entire metabolic process is very unstable, and at this time some slight influence, say of the thyroid and perhaps also of the hypophysis, is able to furnish the stimulus necessary to upset this process and so to initiate the series of vital phenomena which we call metamorphosis.

From the point of view of age, it may be considered that the growth of the body of the thyroidless larvae as a whole, or of most of their organs is precocious, since in a given time they grow more rapidly than the control larvae. The skeleton as a whole and the brain grow more slowly than those of the controls. If thyroidless larvae are compared with control larvae of the same size regardless of age, then the only very precocious growth noticeable is that of the hypophysis.

#### *Organs and method of comparison*

The following figures representing the condition of the organs of the animals studied are all camera-lucida drawings of the organs removed in autopsies or studied in microscopical sections. The number of complete autopsies performed was sixty-nine. In addition there were done about forty partial autopsies. In addition to these, many larvae and frogs were sectioned serially. For comparison of the organs, outline drawings were made and the drawings afterward were compared directly. Measurements were made of the various diameters of those organs of regular contour, but most of the organs are so irregular in outline that such measurements are of little or no value, and the accompanying pictures give a much better idea of the conditions obtaining in most of the organs than would any tables, no matter how carefully prepared. It should be noted that the accompanying figures are drawn from specimens which were carefully chosen as representing the condition as near the average as possible.

The organs are in most cases too small for direct volumetric determinations. Allen ('18) and Rogers ('18) have used the product of the three principal diameters of an organ to represent its volume, but this is not even approximately correct, because of the great variation in the shape of most organs of larvae and

frogs. If two glands of exactly the same size but of different shapes are measured by this method, the volumes determined might differ greatly, while two others of different shape and size might seem to have the same volume. This irregularity in the form of the hypophysis, for instance, is shown in our own figures and in those of Rogers on page 605. Rogers states that the same relative error would apply to all the glands so measured, but he used so few specimens that no two of the glands were of quite the same shape, and hence different errors have been made in his measurements of different specimens. In far greater error are Rogers and Allen when they attempt to establish the relative volume of an organ by dividing the product of its three principal diameters by the length of the animal's body, for the volume of a body varies as the cube of a linear dimension. Moreover, in larvae of different sizes the proportions of the body vary considerably and the difference in the size (volume or weight) of two animals is not directly proportional to the difference in their body length. This is especially true where one attempts to compare tadpoles and frogs. It may be noted in figures 12 and 16 that the length of the real body of the larva (excluding the anal canal) is nearly the same as that of the resulting frog and yet the larvae are from two to three times as large as the frogs produced from them (table 2). It may be noted also that the larger thyroidless larvae have relatively long tails, and hence their size is not directly comparable with that of control larvae on the basis of length of body. Still further, larvae contain relatively much more water in their tissues than do frogs.

Another source of error in work of this sort is that the animals vary in the amount they shrink during fixation. Given two tadpoles fixed in the same fixative, one may shrink nearly twice as much as the other, and hence with a small number of specimens large errors may be introduced in attempting to estimate volumes of structures. Where different fixatives are used, still greater differences in shrinkage result. These animals are so small that unless they are fixed, many of the organs cannot be properly dissected or easily handled, and will undergo post-

mortem changes that entirely alter their volume and shape. There is no practical method by which the exact volumes of these small organs can be determined. One could section them and then calculate the volume of each section by use of a planimeter, and in this way, except for large errors due to shrinkage, estimate approximately the volume of the organs. However, in the case of most of the organs the variability is so large and the actual differences between the control and thyroidless groups are so slight that the result to be obtained would not justify the effort required. We are certain that only large differences in size of glands of these larvae can be detected by any known practical method, and that any small differences noted are as likely to be due to errors of measurement as to the experiment itself.

*Brain.* Figures 22 to 27 show the general character of the brain in the various animals. The brain in the thyroidless animals appears to be relatively small and undifferentiated when compared with the brain of controls of the same age. Comparison of figures 24 and 25 shows the difference that exists between most of the control and thyroidless animals. In the former the brain is practically the same shape and size as that of the frogs immediately after metamorphosis (fig. 23), whereas the brain of the thyroidless larva, especially in the telencephalon, thalamus, optic lobes, and cerebellum, is shaped very much like that of a very small control larva (fig. 22). This undeveloped condition of the brain of the thyroidless larvae may be noted in older and larger specimens. Gradually, however, the brain of these specimens tends to become differentiated (fig. 26) and finally it assumes a condition practically the same as that of the young frog (fig. 27) except for the shape of the anterior part of hemispheres. In some of the large thyroidless larvae (fig. 26) the optic lobes are relatively short, and of a consequence the cerebellum bulges dorsally and hence appears relatively larger than in the other figures shown here, but the difference in size is apparent rather than real. In the still older large thyroidless larvae (fig. 27) the optic lobes are relatively longer than in the former (fig. 26) and overhang the cerebellum, partially



hiding it and pressing upon it so that it becomes more flattened and appears to become smaller.

Allen ('18) noted this early lack of differentiation of the brain of the thyroidless larvae, but did not observe the changes which occur later. It is to be noted that this differentiation of the brain is independent of the changes that occur normally during metamorphosis. The reason for this slow growth of the brain of the thyroidless larva is not evident. In control and thyroidless larvae of the same size the brain in the latter is usually, though not always distinctly smaller, but it is always less highly differentiated in the thyroidless than in the control larva during and for some time after metamorphosis of the control. The brain during metamorphosis, loses very little, if any, in size and hence changes greatly in relative size; so that, relatively, a normal larva has a much smaller brain than a frog, and a thyroidless larva a still smaller one.

*Eyeballs.* The eyeballs of the metamorphosing controls are of about the same size as those of the corresponding thyroidless larvae. During metamorphosis the eyeballs remain about the same size as before or perhaps gain slightly, so that a frog has relatively larger eyes than a control or thyroidless larva. In the latter, however, the eyeballs continue to grow and this difference is somewhat decreased. During metamorphosis the head narrows and the eyeballs approach each other medially, but as the brain loses but little if any in width the optic nerves must be obliged to shorten (compare figs. 13 and 17).

*Heart.* The heart (figs. 28 to 31) has about the same proportions in the control and thyroidless larvae, but varies in size as seen in autopsies on account of varying amounts of enclosed blood. During metamorphosis in the normal animal the heart loses a little in size, but less than the body as a whole. In the older thyroidless larvae, the heart increases in size while the body is growing.

*Liver.* At the time of metamorphosis the liver is very irregular in outline (figs. 32 and 33). A coil of the intestine half embedded in it increases its irregularity. The groove made by the intestine is indicated in the figures. The liver consists of three principal

lobes and minor subdivisions. The organ varies in size considerably. Before the time of metamorphosis it is clearly smaller in the thyroidless than in control larvae of the same size but older. The average difference is slightly less than that indicated by figures 32 and 33. This difference in size possibly indicates that in the thyroidless larvae the metabolism is less rapid than that of the controls, since the size of the liver is ordinarily proportional to the rate of metabolism (Hoskins, '16). The thyroidless larvae grew more rapidly than the controls, however, and one would expect them to have large livers.

During metamorphosis the liver and gall bladder decrease in size as much as or more than the whole body (fig. 34), as is to be expected since the young frog has a lesser total metabolism than the larger tadpole from which it is transformed. Changes in form of the liver also occur during metamorphosis (fig. 34). The groove formed by the coil of the intestine that was embedded in it disappears and the entire organ becomes more compact. The three lobes become very distinct. The liver grows to a considerable size in the larger thyroidless larvae (fig. 35) both actually and relatively, and there is a very definite approach on the part of this organ to the shape of the frog's liver (fig. 34). The three lobes become well marked, but one is still grooved by the coil of the intestine already referred to. After the thyroidless larva has ceased active growth, the liver tends to become smaller so that in the second year of its life the thyroidless larva has, in general, a relatively smaller liver than thyroidless larvae of the first season of the same size of body. This decrease in the size of the liver that occurs in the older specimens is probably accounted for by a decreased metabolism.

*Hypophysis.* The hypophysis of the frog is composed of a glandular portion developed from the ectoderm and a non-glandular part, the infundibulum formed by the brain. The former portion becomes subdivided into three secondary lobes corresponding in position with those of fishes. We shall refer to these secondary lobes by the terms generally used for hypophysis of fishes, namely, anterior, superior, and inferior lobes. Of these three lobes, the inferior is the largest, shows the earliest

histological differentiation, and appears to be the most actively secretory portion of the entire hypophysis. Atwell ('18) names the lobes of the frog's hypophysis pars tuberalis, pars intermedia, and anterior lobe proper. These names correspond to those used in higher groups of animals, but we have used the terms anterior, superior, and inferior as being more descriptive. The anterior lobe is very inconstant in size and shape. In some cases it is forked, and it may be completely subdivided into two smaller lobes in later larval stages and frogs. Its most usual arrangement is that of a thin plate of cells closely adherent to the infundibulum, anterior to the inferior lobe. At times it is completely buried within the inferior lobe. It showed no particular secretory activity in our microscopical preparations and may not be of much importance, although its size was usually proportional to that of the body of the animal. After studying and drawing with a camera lucida the hypophyses of more than ninety larvae and young frogs and after careful comparison of the drawings with each other, we selected figures 36 to 45 as properly representative of the hypophyses of the different groups of animals. The glands were studied microscopically in other specimens also. The hypophysis is too irregular in outline to permit accurate determination of its volume from measurements of the three principal diameters, as was attempted by Rogers ('18), and is too small for accurate weighing or direct volume determinations.

It is obvious that glands vary in size with the size of the body in animals in the same stage of development. Rogers' three groups of animals were not of the same size nor of the same shape, and since he failed to reduce his measurements to comparable figures, his conclusions are unwarranted and, as we shall show, are mostly incorrect. His experimental larvae, judging from differences in *R. sylvatica* with which we have worked, must average nearly, if not quite, twice as large as his control larvae, but their body length is only about 25 per cent greater (Rogers, p. 594). Dividing the volume of the glands by the body length makes the glands of the longer animal appear relatively large.

As is shown by figures 36 to 39, the hypophysis is larger in thyroidless larvae than in controls of the same size at the time of metamorphosis. These figures represent fairly closely the difference in size between the hypophyses of our control and experimental larvae at this stage of their development. The hypophysis and especially the inferior lobe may be considered to have hypertrophied in the thyroidless larva as compared with that of the control of the same size (figs. 36 and 38). The difference is slightly greater than is indicated by the two drawings, because the thyroidless larva (fig. 36) was slightly smaller than the control (fig. 38). The difference, however, is much less than that described by Rogers ('18), who endeavors to compare the hypophysis (or part of it) of control larvae with that of thyroidless larvae much larger. His method of 'correction' for body length introduces a large error into his figures, as noted above. In Rogers' summary (p. 594) he states that after correcting for difference in the size of the animals, the hypophysis of the controls is represented by the figure 152 and that of the thyroidless by 776. This would indicate an hypertrophy in the latter of some 300 per cent, a figure obviously incorrect. Rogers leaves out of his summary (p. 594) the hypophysis of his thyroidless larva no. 4, with no explanation for an omission which changes greatly the 'average volume' of this group, as this particular hypophysis is but little more than half the average volume. The hypophysis of Rogers' control larva no. 10 (which is about the same size as the thyroidless larva no. 9, p. 594) has nearly one-half the 'volume' of the hypophysis of the thyroidless animal. The difference between these two is less than the difference between the extremes in either group of larvae. As a matter of fact, the hypophysis of the thyroidless larvae has less than two times the volume of that of controls of the same size in our experiments (figs. 36 and 38). If there were no real difference at all between the two, Rogers' method of 'correction' would still show an apparent difference in all cases in which the glands of animals of different sizes were compared.

In our larger thyroidless larvae, the hypophysis (fig. 42) is relatively larger than in the smaller thyroidless larvae, so that



a still greater hypertrophy seems to be indicated than in the hypophysis of the smaller animals, and especially is this true of the inferior lobe. The shape of these larger hypophyses varies. In some specimens the superior lobe is not so completely covered by the inferior as in figure 42, and this increases the irregularity of the outline of the gland and also the error in any attempt to estimate its volume by multiplying together its three principal diameters. This irregularity is indicated in figure 44 of the hypophysis of a thyroidless larva that survived the second season. In such older larvae the large proportions of the hypophysis (fig. 44) are pretty well maintained; but we have autopsied only three of these specimens, and their hypophyses are quite variable in size, so we cannot be certain whether or not there is any further change in the relative volume of the gland during the winter and second spring and summer. The three hypophyses just referred to are, however, all smaller than those of much younger larvae of the same size.

It cannot be determined how much of the overgrowth of the hypophysis in larger thyroidless larvae is a true hyperplasia, because of the lack of a proper basis of comparison. If the control larvae could in any way be induced to grow as large as these larger thyroidless larvae, their hypophyses might also become relatively larger than those of smaller tadpoles. It is well known that the relative size of most organs of an animal changes as the animal becomes larger, and age changes also are evident.

In a given time the body of the thyroidless larva becomes larger than that of the control and its hypophysis increases greatly in size, but it must be admitted that the great increase in the size of this organ may be due to the factors' causing overgrowth of the entire body rather than that the reverse is the case. However, from what is known in other animals and from the fact that the hypophysis in the larger thyroidless larvae is relatively larger than in the smaller thyroidless or control larvae, it is probable that the overgrowth of the hypophysis accounts partly at least for the overgrowth of the body as a whole.

As stated above, the principal lobe of the hypophysis appears to be the inferior. This lobe is actually wedge-shaped, as in-

dicated in the drawings, and this adds to the difficulty of estimating the volume of the gland. During metamorphosis the actual size of the hypophysis remains practically unchanged, but there may be a slight decrease. Any difference in size to be noted is less than the normal variability and less than the experimental error in any attempt made to determine the size. Figure 40 shows a representative specimen of average size of an hypophysis after metamorphosis. Some of the glands of these young frogs were smaller than those of the control larvae, but more of them were larger. Rogers ('18) states that the hypophysis shrinks during metamorphosis, but his data (p. 594) do not support his conclusion. He apparently examined only three specimens of the young frog's hypophysis, of which one (no. 13) is larger than that of three out of six control tadpoles, another is larger than one of his controls, and the difference between the average of his experimental and control specimens is much less than the variability shown within either group, and is much less than the experimental error of his method of comparison. Rogers' hypophysis no. 14 is only one-third as large as his no. 13. His specimens are so few in number that the average size is determined by the accidental selection of material. Selecting at random he might have examined three specimens of the size of no. 13 and drawn the conclusion that there is no shrinkage at all during metamorphosis. In the large number of specimens which we examined there was the same sort of variability shown. However, a slight growth or a slight shrinkage of the hypophysis during metamorphosis would be of no importance, because, as shown in table 2, there is a shrinkage of one-half to two-thirds in the size of the entire animal during this process, so that the hypophysis increases considerably in relative size at this time. Hence it is obvious that a young frog has a much larger hypophysis in proportion to the size of the entire body than does the larva from which it is transformed.

It so happened that in the first larvae we autopsied the hypophysis of the female in every case was larger than that of the male, and we so reported the matter (Hoskins and Hoskins, '18). In a much larger series of autopsies we have since observed that

the male hypophysis is often larger than that of the female of the same size, so that our first findings were the result of chance variation. From our recent observations it is evident that there is considerable variability in the size of the hypophysis and no constant size difference between the sexes exists.

**Microscopic structure.** The three lobes of the hypophysis are well shown in the figures 56 to 59. In smaller larvae the three lobes consist of closely packed undifferentiated cells irregularly round with granular cytoplasm and containing nuclei of corresponding shape. This condition is retained normally until nearly the time of metamorphosis (fig. 56). At the time of metamorphosis the cells of the inferior lobe have usually begun to form cords which are separated by connective tissue (figs. 57 and 59), but the gland in some other cases is still undifferentiated even after metamorphosis (fig. 60).

In larger thyroidless larvae at the time of metamorphosis of the controls the inferior lobe (fig. 58) has already been divided into cords, but the other two lobes are not differentiated. The gland is relatively larger in these thyroidless specimens than in the controls of the same age, and most of the hypertrophy is confined to the inferior lobe. After cords form in the inferior lobe there are found two kinds of cells there. One is of the undifferentiated type described above and the other is somewhat columnar with cytoplasm finely granular and eosinophilic and containing a round or oval darkly staining nucleus. A few of these eosinophilic cells are found in the controls just before metamorphosis and a larger number in the thyroidless larvae of the same age (figs. 61 and 62). At this time the anterior and superior lobes consist of cells of indefinite shape (figs. 61 and 62).

In older thyroidless larvae (fig. 63) the inferior lobe of the hypophysis contains a larger percentage of eosinophilic cells than it does in younger larvae. Most of these cells are irregular in shape, but some are columnar. Their cytoplasm is often so nearly homogeneous as to have the appearance of colloid. The non-eosinophilic (chief) cells are much more abundant than those of the eosinophilic type. In these older larvae the cells of the

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superior lobe of the hypophysis are irregularly round or columnar in shape with basal nuclei that are smaller than those of the cells in the inferior lobe. The anterior lobe consists of cells with very little cytoplasm and with large nuclei about the size of those of the inferior lobe or slightly larger.

The hypophysis may be the source of the stimulus which causes the thyroidless larvae (with enlarged hypophysis) to grow more rapidly than the controls, although the hypertrophy of this gland was not noticeable until after the time at which the difference in the rate of growth began. However, the variability of size of the hypophysis is considerable and it is also true that the secretory activity of a given organ is not necessarily exactly proportional to its size. An organ with a rapid secretory rate would produce more secretion than a somewhat larger organ with a slow rate. It is also true that the chemical nature of the secretion of the hypophysis may be changed by removal of the thyroid.

*Thymus.* The thymus in frog larvae is paired, one of the pair located on each side laterally between the eye and ear and dorsal to, but near the gills. In the change of form through which the animal goes at metamorphosis, the depressor maxillae grows posteriorly between the eye and the thymus and pushes the latter posteriorly and ventral to the junction of the jugal, pterygoid, and tympanicum.

The thymus reacts to thyroidectomy in much the same way as all other organs except the hypophysis. It is not affected at all up to the time of metamorphosis or at least no effect can be determined either in size or structure. Our figures 46 to 49 represent a few of the shapes this organ assumes, and others are shown by Rogers (p. 605). This variation is even greater than that of the hypophysis, and the objection made to Rogers' method of calculating the volume of the latter gland applies here. He states that the errors made in the calculation for different groups counterbalance each other, but, as he shows on page 605, there were relatively few glands measured, and the typical shape of the gland in the different groups is not the same. Some of the thymi are nearly cylindrical, some (especially after



metamorphosis) are more nearly spherical, and still others more closely resemble parallelopipeds. The volumes of bodies of these shapes vary greatly when considered in relation to their three principal dimensions, and hence they are not comparable.

Figures 46 and 47 are representative of the thymus as it exists in operated and control larvae of the same size at a time just preceding metamorphosis of the latter. Any difference between the total volume of the two thymi of the thyroidless larva (fig. 46) and that of the control (fig. 47) is no greater than the normal variability of the thymus within either group. During metamorphosis the thymus changes in shape and decreases in size (fig. 48). Microscopic examinations showed no difference in compactness or size of the cells of the thymus after metamorphosis, and hence this decrease in size of the gland is due to an actual decrease in the thymic tissue. The change in shape of the thymus at this time is due to change in the pressure from the surrounding structures. There is a decrease in the volume of the thymus during metamorphosis, the young frog has relatively a larger thymus than the control larva, since the volume of the larva decreases more at this time than does that of the gland. This fact is overlooked by Rogers, who shows (p. 595) that after he had 'corrected' the combined 'volumes' of the thymi these glands were relatively about twice as large in his control tadpoles as in the young frogs.

Rogers ('18) compares the thymus of various groups of animals with a single small thymus taken from a single animal (no. 4, p. 594) and draws general conclusions from this (pp. 596 to 598). He overlooked the fact that this particular gland is abnormally small, and the other one of the pair which was not measured might well have been quite large, as may be seen to be the case in several of his specimens (p. 594) and as is very evident in our own material. Had Rogers lost or destroyed the right thymus of animal no. 4 instead of the left thymus as happened, or had he killed his animal no. 8 instead of his no. 4 at his time, his conclusions might have been greatly different from what they were, in regard to the thymus. As a matter of fact, the average size (volume or weight) of young frogs is from three to five times

less than that of thyroidless larvae of the same age and, as shown in our material, the relative volumes of the thymus of the two groups of animals are about the same. The variability of the thymus is so great, as shown by our own and Rogers' material, that it cannot be stated with certainty that the thymus is relatively larger in young frogs than in the larger thyroidless larvae, although probably it is so. It is relatively larger, however, in frogs than in thyroidless larvae which are the same size as the tadpoles from which the frogs have been transformed. In our 1918 series some of our thyroidless larvae attained nearly their maximum possible size in June and July, but their thymus glands were not relatively smaller than in those 1917 larvae which reached this size in the autumn or winter, so the age relationship emphasized by Rogers on page 597 does not exist. Rogers' graph on page 599 is very misleading. It indicates that normal tadpoles have thymus glands several times as large as those of the thyroidless larvae of the same age, whereas such is not the case. The difference in the two curves at this point is due to the use of the single small thymus of the thyroidless larva referred to above and does not represent the true condition. Both these graphs and those on page 598 are based upon few incorrectly measured volumes incorrectly standardized.

Three of our thyroidless larvae which survived the second season were autopsied and of these, two had thymus glands that were relatively somewhat smaller than the general average of the group of younger large thyroidless larvae, but the thymus of the third larva was fully as large as that of any of the younger specimens. This shows that the thymus will continue to remain large in some of these animals, at least, that are kept in the larval condition for more than one year. Frogs at all ages have actually a smaller thymus than the large thyroidless larvae and the large growth of the gland in the latter might be considered as an hypertrophied condition, but it must be admitted, as in the case of the other organs of these larvae, that there is no standard of comparison for them. If the control tadpoles could be induced to grow as large as these experimental larvae, their thymi might also become large. Figures 47 and 49 show, however,

that the thymus is relatively larger in the thyroidless larvae than in control larvae just about ready to undergo metamorphosis.

**Microscopic structure.** The thymus consists of a medulla of varying proportions and a cortex. There is to be seen no constant histological difference between the thymus of control and thyroidless larvae at the time of metamorphosis (figs. 64 to 67). The cortex consists almost entirely of small lymphocytes. The medulla contains both small lymphocytes and large thymic cells with large and often irregular nuclei and granular eosinophilic cytoplasm. The percentage of large cells varies in thyroidless larvae of different ages and in the control larvae and frogs; there is no marked difference noticeable in this respect between the thymi of the various groups of animals.

During metamorphosis the entire thymus shrinks in volume, but does not appear to be changed in structure. The cells are not smaller after metamorphosis than before. In some specimens the cells are placed more closely together than before, but in other cases this is not so. In no case can this account for the entire shrinkage of the organ, and hence the shrinkage is not due merely to loss of fluids.

*Epithelioid (parathyroid) bodies.* The epithelioid bodies of the control and thyroidless larvae of the same size are themselves the same size within limits of normal variability, as shown by comparison of camera-lucida drawings and direct comparison of the organs themselves. Figures 50 and 51 seem to show that the volume of the epithelioid bodies of the experimental larvae are slightly larger than those of the control larvae of the same size, but in other specimens the reverse is true. In larvae these bodies are usually spheroid in shape and their volume can be measured approximately with the formula  $\frac{4}{3} \pi r^3$ . This was done, but the measurements do not show any constant difference in size between the epithelioid bodies of thyroidless and control larvae of the same size.

During metamorphosis there is practically no change in the absolute size of the epithelioid bodies, except possibly a slight decrease of the average, but since the volume of the animal decreases at this time, as stated above, the relative size of the epithelioid bodies is increased considerably.

Figure 53 shows a ventral view of the epithelioid bodies of a thyroidless larva of the same age as the control, but of nearly twice the volume. These bodies are actually about three times as large as those of the control larvae, and relatively they are somewhat larger than those of the controls.

In still older and larger thyroidless larvae the epithelioid bodies are relatively larger than in those just described. Their volume is not easily measured, however, because they become flattened somewhat and the formula for measuring the volume of a sphere is no longer applicable to them, although they might be measured as ellipsoids.

**Microscopic structure.** Since the epithelioid bodies are believed to correspond to the parathyroid glands of higher animals, it would not be unlikely that they should undergo some modification after removal of the thyroid, but no structural change is evident in our material (figs. 68 and 69). The epithelioid bodies of control and thyroidless larvae shown here are seen to be composed of cells with large darkly staining round or oval nuclei and finely granular cytoplasm. The cell boundaries are almost indistinguishable. The body has a well-defined capsule. Its blood supply is poor and this together with its cytological appearance argues against any great secretory activity on its part. After metamorphosis and in our older thyroidless larvae the structure of these bodies seems to be the same as in the normal larvae.

**Thyroid.** The thyroid is missing from all the experimental larvae which did not metamorphose, but it was studied in the normal tadpoles and young frogs. Normally, there are two thyroids, located on the ventral side of the hyoid at its posterior border near the median plane. We found one specimen in which the two thyroids were connected by an isthmus of thyroid tissue as in mammals. In another specimen (fig. 54) the two were nearly joined together. Comparison of the thyroid of twenty control larvae and twenty-five young frogs shows that the volume is practically unchanged during metamorphosis (figs. 54 and 55). This is an important point, since removal of the thyroid prevents the occurrence of this transformation. Neither the



size of the gland nor the histological structure in normal specimens indicates that it is more active during metamorphosis than before. However, when we consider that the thyroids do not decrease appreciably in actual size during metamorphosis, whereas the size of the entire animal decreases at this time from one-half to two-thirds, the result is that relatively, the thyroid becomes continually larger during this transformation of the body, and the young frog has relatively much more thyroid tissue than the larva. One precocious operated animal (fig. 20), which metamorphosed earlier than the controls and at a smaller size, had regenerated thyroids relatively larger than normal; and Gudernatsch ('14) and others have shown that feeding thyroid to tadpoles hastens their metamorphosis. In another experimental animal, however, the thyroid that regenerated and permitted metamorphosis consisted of a single gland with a volume less than one-fourth the combined volume of the two thyroids in the normal animal, and this regenerated gland did not have a normal cellular structure nor very much colloid (fig. 70). In the other experimental larvae in which the thyroids regenerated the two glands varied in size considerably.

These observations together with those of Gudernatsch indicate that, although an abnormally increased amount of thyroid hastens metamorphosis of the frog larva and although this transformation will not occur if the thyroid is entirely removed (and the larvae kept on a normal diet), still a thyroid gland much smaller than normal was able to furnish the stimulus necessary to bring about metamorphosis. This small thyroid may have produced its secretion at a rate more rapid than normal, but the need for added secretion ought to have caused an hypertrophy of the gland.

The part played in metamorphosis by the thyroid secretion has already been discussed. From the result of the experiment on forced metamorphosis in a thyroidless tadpole, it does not seem that secretion is necessary for the atrophy of parts which occurs during this process.

Microscopic structure. The two thyroids of *R. sylvatica* are well developed normally and contain colloid in larvae of 15 to 20

mm., at about the time when the corresponding thyroidless larvae begin to grow more rapidly than the controls. The two phenomena may, however, be only concomitantly and not causally related, and the rapid growth of the thyroidless larvae may be due to the hypertrophy of the hypophysis or to some other cause, and not to the absence of thyroid secretion.

The thyroid consists of large follicles filled with colloid and lined by cuboidal epithelium with large round nuclei and granular cytoplasm (figs. 73 and 74). There is a capsule, but very little interfollicular tissue. During metamorphosis there is no change in the structure of the thyroid, but there occurs a large increase in relative size.

Figure 70 shows a section of a regenerated thyroid of a metamorphosed operated animal which is much less than half the volume of a normal thyroid. Since only one of these glands was present, the total volume of thyroid tissue was much less than one-fourth of that of a normal animal in the same stage (fig. 71). The structure of this regenerated thyroid was not normal. The gland consists of relatively few large irregular follicles containing colloid. The epithelium (fig. 72) is columnar with oval nuclei and granular cytoplasm. In the cell can be seen droplets of colloid, more numerous than in the normal thyroid (fig. 73). This regenerated gland was located beneath the tongue, considerably anterior to its normal position. This serves as a caution for the use of great care in examining frogs transformed from larvae that have had the thyroid removed. This small regenerated thyroid shown here might easily have been overlooked and the conclusion that the thyroid is not needed for metamorphosis would then have been drawn.

Another unusual regenerated thyroid is shown in figure 75. This gland is one of two which were normal in structure but large in size. The size of the thyroids of the corresponding control larva is indicated in figure 74. It is interesting to note that the larva with this large regenerated thyroid metamorphosed at a size of about one-half normal and was transformed into a frog of one-third the normal size. The total actual volume of this thyroid was not as great as that of the large control larvae

at the time of metamorphosis, but in proportion to the size of the animal it was several times larger than normal (compare figs. 71, 74, and 75), and this fact probably accounts for the precocious metamorphosis similar to that which Gudernatseh ('12) obtained by feeding thyroid.

*Spleen.* The variability in the size of the spleen in amphibia as in mammals is very great and seems to follow no definite laws. In our normal larvae and frogs the variability of this organ was fully 100 per cent. The 'average' size of the spleen in the control larvae just before metamorphosis was about the same as that of the thyroidless larvae of the same size (figs. 79 and 80). During metamorphosis the spleen changes little, if any, in absolute size except perhaps a very slight increase; but its relative size increases in about the inverse ratio as the change in the size of the body, which, as stated above, decreases by one-half to two-thirds. The proportions of the spleen in the larger thyroidless larvae are about the same as in the control larvae and hence somewhat less than in young frogs, but, as shown in figures 83, 84, 86, and 92, the variability is very large.

The spleen becomes differentiated in structure by the time the larva is 26 mm. long or even before this time (fig. 76). The cellular structure consists of a dense reticulum containing small lymphocytes and large splenic cells with more cytoplasm than the lymphocytes. The nuclei of the large cells are usually lightly stained. The nuclei in the spleen are often irregular in outline and many seem to be dividing by simple fission.

There is no particular cellular difference between the spleens of control and thyroidless larvae (figs. 77 and 78).

*Kidneys.* The kidneys in our various groups of animals resemble in general the liver and the heart in changes of relative size. Given a control and thyroidless larva of the same size (figs. 79 and 80), the kidneys of the two are the same size, within the limits of normal variation.

During metamorphosis the size of the kidney decreases considerably, but the decrease is proportionally less than the loss in the size of the entire animal, so that a young frog has relatively a larger kidney than a normal tadpole (figs. 79 and 81).

The kidneys, liver, and heart of mammals are known to vary in size with the rate and amount of metabolism, when the animals are in a given stage of their life, and the same is probably true of these organs in amphibia. The renal excretion of a young frog is doubtless less in amount than that of the much larger tadpole from which it is transformed, and smaller kidneys are needed by the frog than by the tadpole, hence the decrease in size suffered by the kidneys. The fact that the young frog's kidneys are relatively larger than those of the tadpole may indicate that the frog's renal excretion is relatively greater in amount than that of the tadpole. We have made no study to determine whether the loss in the size of the kidney during metamorphosis is due to a decrease in the number of tubules or in the length or diameter of these structures. In the thyroidless tadpoles which ultimately become from two to three times as large as the control larvae, the kidneys are relatively larger than those of the latter (figs. 79 and 83), and are correspondingly larger than those of the smaller thyroidless larvae (fig. 80). They are of about the same relative size as those of young frogs. We have no data by which to compare the kidneys of older frogs with those of the older thyroidless larvae. In the thyroidless larvae which were autopsied at the end of the second season the kidneys were relatively smaller than in those thyroidless larvae autopsied the preceding autumn when they were at the end of a period of rapid growth.

We made no observations on the fat bodies beyond noting that they vary in size, do not change much, if any, during metamorphosis, and become large in the large thyroidless larvae.

*Gills and lungs.* The size of the internal gills of control and thyroidless larvae is proportional to the size of the body. In the older thyroidless larvae the gills grow in absolute size much larger than they ever are in normal larvae and persist as long as the larvae are kept alive. Thus, structures which normally exist during only a few weeks or months are maintained more or less indefinitely.

The lungs normally become functional by the time larvae are half-grown. The larvae can be seen to start deep in the aqua-



rium and swim rapidly toward the surface, their momentum enabling them to reach the air which they breathe in. This air is soon expelled again and the process repeated. Not only do the normal larvae which are later to become frogs use their lungs in this manner, but the thyroidless animals which are to remain in the larval state do the same. As they later become larger their lungs become very large, extending to the posterior limit of the body cavity. These older thyroidless larvae breathe in air less frequently than they do at the time of normal metamorphosis, but when they are autopsied their lungs are always seen to contain air. They are not dependent on their internal gills for respiration, as was seen when one of them was placed in a moist chamber. In this situation the animal lived for two days, breathing irregularly. Ultimately it must starve, as it cannot move about or feed as in normal circumstances.

*Intestines.* As noted by Allen ('18), the intestinal tract of the thyroidless larva does not shorten as does that of the normal larva in metamorphosis, but continues to increase in length and diameter with the growth of the animal. As shown in figure 35, the groove in the liver which is made by the gut persists in all of the thyroidless larvae. The variability in the length of the intestine has already been discussed (Allen, '18, p. 505).

*Adrenals.* The adrenals in amphibia are, unfortunately, so diffusely scattered along the kidney as to render impractical a study of their size. In mammals, we have shown (Hoskins, '16) that the size of the suprarenals tends to increase, with increase in the size of the liver, kidney, and heart produced by changes in metabolism that are in turn brought about by thyroid feeding. Somewhat similar interrelations should exist in amphibia, although these structures are not in quite the same condition as in mammals.

*Skin.* The only differences in the skin to be noted in our various animals is that in many cases, although not in all, the skin of the thyroidless larva tends to contain more pigment than the normal amount, and these larvae thus appear darker than the controls. The integumentary glands developed normally in thyroidless larvae.

*Ovaries.* The left ovary of our animals (*R. sylvatica*) is nearly always larger than the right ovary. In control and thyroidless larvae of the same size the ovaries are of similar volume within the limits of normal variability (figs. 79 and 80). These thyroidless larvae are younger than the controls, but of the same size. In larger thyroidless larvae of the same age as the control larvae the ovaries are larger in absolute size than those of the controls, but in relative size they are about the same as the controls, or possibly smaller.

During metamorphosis the ovaries of the normal larvae (fig. 81) change very little in absolute size, beyond perhaps a slight increase; but the decrease of one-half to two-thirds in the size of the body that occurs at this time results in a corresponding increase in the relative size of the ovaries in young frogs, which thus have relatively much larger ovaries than do either the control or thyroidless larvae.

In the older (and larger) thyroidless larvae the ovaries reach their maximum size at about the same time that the body ceases growing. Figures 83, 84, and 85 show that in July, 1918, the ovaries of some 1917 larvae are very little larger than in the preceding December and October, although the oocytes are larger in the largest animal. The larva in the meantime had also grown slightly. The actual size of the ova will be discussed later. The size of the ovary varies considerably in different larvae of the same size and age, but those shown in the three figures represent the average condition in our 1917 specimens. The third dimension of the ovaries is not well shown in the drawings, but it was studied by the method described above.

In our 1918 animals which grew very rapidly the ovaries at the normal time of metamorphosis had not grown quite as large as in the 1917 series which grew more slowly (compare figs. 81 and 86), but the difference between them is not great. Figure 80 shows an ovary of a large but very young 1918 thyroidless larva which still retains the flattened larval shape, although the animal itself was as large as some of the 1917 larvae which were much older and in which the ovaries were further advanced in their development. Hence it is apparent that the time element

of the growth does have some influence on the rate of development of the ovary, but the effect is not different from the effect upon various other organs and is not characteristic of the gonads.

Allen ('18, pp. 513, 514) emphasises the fact that in estimated volumes the ovaries of old thyroidless larvae are about four times as great as those of young frogs, but he does not note that such larvae themselves are from three to six times the size of such frogs. Hence in relative size the frogs have the larger ovaries. The growth of the ovaries in absolute size in these thyroidless larvae resembles the growth of other organs, such as the liver, thymus, heart, etc.

Other evidence of the fact that rapidity of growth plays a part in the development of organs of animals in similar stages of development is shown by the ovaries of the newly metamorphosed frogs of the 1917 and 1918 series. In the former, which as stated above grew more slowly and did not metamorphose until August, the ovaries are slightly further advanced in development than in the latter, which grew more rapidly and metamorphosed in June. In the former the oocytes are evident on the surface of the ovary and the ovary has an adult shape, whereas in the latter the ovary is often still flat and oocytes are but very faintly indicated on the surface. It is also true, however, that organs other than the gonads were further advanced in these 1917 frogs than in the younger 1918 frogs. Further, as stated above, if the control larvae could in some way be induced to grow as large as these thyroidless larvae their ovaries would doubtless also become larger before metamorphosis.

An increase in size is a matter of growth rather than of differentiation. The latter process may be said to be complete in the ovary when maturation occurs, as the eggs leave the ovary. Maturation has not been seen in the ovaries of our thyroidless larvae, even when they have been kept alive through the second season, and therefore the experiments do not prove that the ovaries are independent of the soma in their development. We cannot agree with Allen's statement that thyroidectomy demonstrates a sharp difference between the gonads and the remainder

of the body, as other organs are equally independent in their development.

Oviducts did not develop in the animals kept through the second season, so sexual maturity cannot be said to have been reached, and the condition is not true neoteny, according to the present definition of that term.

**Microscopic structure.** Figures 94 to 103 represent the microscopic structure of the ovaries of animals in various stages of development. Synapsis begins often in normal larvae when the animals are but 25 mm. long and only about one-fourth grown. The great variation in the time of synapsis prevents a determination as to whether thyroidectomy stimulates this process directly, although it is clear that an indirect influence is exerted by the rapid growth of the thyroidless larvae.

The question of the relation between the differentiation of gonads and that of the body is very complex. While the former is not entirely independent of the latter, it is far from being dependent on it, as a study of the accompanying figures (94 to 103) shows. In the 1917 animals, which did not receive an optimum food supply and which grew slowly, the ovaries at metamorphosis showed well-developed oocytes  $120\ \mu$  in diameter (fig. 96). The ovary of a very small precocious frog (fig. 95) was actually smaller than that shown in figure 96, but relatively larger. Oogenesis in it had progressed faster than in the normal animal, but the oocytes were smaller, being not more than  $75\ \mu$  in diameter. The control specimen of the precocious frog was at this time a larva 40 mm. long, with ovaries smaller than that of the frog and with oogenesis less advanced.

In contrast with the above relations, it was found in 1918 that in normal larvae at the time of metamorphosis, in thyroidless larvae of the same size, and in young frogs, all of which had grown rapidly, the ovaries were but slightly differentiated (fig. 97) and were smaller than those of the corresponding animals of 1917. The oocytes in the 1918 specimens were not more than  $50\ \mu$  in diameter, about the size of those found in some half-grown 1917 larvae. The problem is further complicated by the fact that in the larger 1918 larvae which grew much more



rapidly than the controls, the ovaries were actually much larger, but relatively smaller, than those of the 1917 larvae of a corresponding stage, and their oocytes were smaller ( $60\ \mu$ , fig. 98). As figure 99 shows, the defective thyroidless larvae which grew very slowly, developed ovaries actually smaller than those of the larger and younger thyroidless larvae of 1918, and the oocytes in these small ovaries were larger ( $130\ \mu$ ) than those of the others. Figure 100 shows the ovary of a large thyroidless larva of the age of the small thyroidless specimen of figure 99, and of nearly the same size as that from which the ovary shown in figure 98 was taken. In the ovary of figure 100 the oocytes are numerous and large ( $180\ \mu$ ). Figure 101 shows the ovary of a large and thyroidless larva of 1917. The oocytes are fewer but larger ( $200\ \mu$ ) than those in figure 100. In figure 102, the ovary of a still older thyroidless larva of 1917, the oocytes are  $250\ \mu$  in diameter. During the second season the ovary and oocytes again increased in size (fig. 103, oocytes  $300\ \mu$ ). The animal from which the ovary in figure 103 was taken was about the same size as the animal whose ovary is shown in figure 100, but five times as old. The latter specimen was about the same size as the one whose ovary is shown in figure 98, but three times as old. The animal referred to for figure 100, on the other hand, was about the same size as that from which figure 101 was drawn, but was only one-fourth as old.

It will be seen that from the animals in our experiments one can deduce no general law governing the development of the ovary, either in size or differentiation of the cells. There is some correlation between the rate of development of the ovary and the rate of growth of the body, but none between the size of the ovary and that of the body, except in a general way. There is no correlation between the stage of differentiation seen within the ovary and its size or its change from the very flat to the oval shape. In general one may say that when the larvae grow slowly the relative rate of differentiation in the ovaries is increased. When they grow rapidly the relative rate of differentiation is decreased, but the difference between these rates of ovarian differentiation is not proportional to the differences in the rate of growth of the body as a whole.

*Testes.* The left testis in our animals is usually larger than the right. In our 1918 series where the size of the testes at various ages was studied we noted that within normal variability the testes of the control larvae before metamorphosis were of the same actual volume as those of thyroidless larvae which were the same size as the control larvae. The testes of the controls were also of the same relative volume as those of larger thyroidless larvae which were of the same age as the controls (figs. 88 and 89).

The testes were studied from camera-lucida drawings much larger than those shown here and were compared also by the method described above, whereby two organs to be compared are placed side by side under a high-powered binocular dissecting microscope and their relative volumes estimated with the eye. One does not try to reduce such volumes to figures, but figures so obtained would not be much more inaccurate than those obtained by multiplying together three principal dimensions of an organ, as is sometimes done. Small differences could not be determined by either method.

The normal variability in the size of the testes is very great. During metamorphosis they do not decrease in absolute size as do many somatic structures, but usually gain very slightly (fig. 90). The decrease in the size of the entire animal during metamorphosis results in a very decided increase (100 to 300 per cent) in the relative size of the testes, so that a young frog has relatively much larger testes than either the control larva from which it is transformed or a thyroidless larva of the same size or age of this control.

In our 1918 animals which grew very rapidly the testes developed actually more rapidly (as did the ovaries) than in the 1917 group, but more slowly in relation to the development of the body as a whole. This was shown by the fact that the testes of the 1918 animals were still in the flattened elongated larval shape even after the period of metamorphosis (figs. 89 and 90), whereas those of the 1917 animals had become ellipsoid like the testes of frogs and of older thyroidless larvae (figs. 91 and 92). In the thyroidless larvae of 1918 the testes had become rounded

in July, so it is probable that those of the frogs would have done so had these animals been kept alive. Hence the difference in the rate of development of the testes between the 1917 and 1918 animals was not proportional to the difference in time required for their development. This compromise indicates that while the testes in their development are not independent of the body, nevertheless they are not entirely dependent upon the rate of differentiation of the body as a whole.

In the larger 1917 thyroidless larvae killed in July, 1918 (fig. 93), the testes were relatively larger than in the control larvae of the stage just preceding metamorphosis. The testes therefore grow relatively more rapidly in the larger than in the smaller animals, but differences exist in the rate of growth of various other organs at different periods of any animal's life, so this is not characteristic of the gonads alone. Moreover, a part of the great increase in the size of the testes is due simply to an expansion of the tubules that does not represent additional testicular tissue. In this expansion the volume increases more than the weight of the testes and prevents accurate comparisons where volumes only are considered.

In age alone the thyroidless larvae are sexually precocious because their gonads develop much more rapidly than those of the controls, but since the entire body grows still more rapidly than the controls it might be argued that this is not true sexual precocity, even through the animals remain in the larval form.

Normally, synopsis occurs in the testis some weeks after metamorphosis (Swingle, '18). In our 1917 animals it had begun in many of the thyroidless larvae of the same age as the controls at metamorphosis, but these thyroidless larvae were considerably larger than the control larvae ever became and hence are not directly comparable with them. In thyroidless larvae of the same size as the controls (but younger) the condition in the testis was the same in both groups, the growth of the testis thus tending to keep pace with the body growth, as does that of other organs. Swingle ('18) noted that inanition retards both bodily and sexual differentiation in frog larvae. The testes do not differentiate in starved animals for the reason that the larvae

do not grow to the size at which differentiation occurs. Their growth tendency being weaker than that of some other tissues (Jackson, '15), they grow less than these other tissues during inanition. In this failure to grow they resemble many other organs.

Like the ovaries, the testes in these thyroidless larvae may be said to be independent of the soma only in the same sense that various parts of the soma are independent of the rest of the body. Differentiation of the testes, as of the heart, eyes, brain, and other organs, is a matter of growth after the anlage is once laid down in the embryo, and in these thyroidless larvae it must be remembered that the general growth process goes on after maturation and after the testes have attained their mature shape. Several organs differentiate before the gonads and others later, so one cannot draw a sharp distinction between soma and gonads, as Allen ('18) does, although the gonads do have some individuality in their growth. Were the gonads entirely independent of the soma, their differentiation in our 1917 animals should have occurred much earlier than it did (in August and September), since in the more rapidly growing 1918 larvae differentiation of the gonads began in June and July. In attempting to compare the testes of the thyroidless larvae and frogs Allen ('18) makes the unfortunate mistake of trying to reduce figures representing volumes of organs to relatively equal values by dividing these volumes by the length of the animal. The error of this method is discussed above.

**Microscopic structure.** In none of the testes of control larvae, or thyroidless larvae of the same size, or of young frogs was synapsis seen to have occurred, so that in these groups of animals the rate of differentiation of the gland could not be determined (figs. 104 and 105). In thyroidless larvae which were the same age as the controls (1917), but larger, synapsis was well under way, and often nearly complete in August when spermatocytes were found in the sections (fig. 107). In the 1918 (rapidly growing) thyroidless larvae spermatogenesis was hastened, but less than the growth of body as a whole. In these larvae synapsis was observed in the testes in July, about a month after the controls had metamorphosed.



None of the 1917 thyroidless larvae were killed in September, but in October the testes of those which were examined were seen to be in an advanced stage of differentiation, with some fully formed spermatozoa (fig. 108). We do not know just when spermatozoa are first produced normally in *R. sylvatica*, but Allen ('18) states that this occurs in *R. pipiens* during the winter, so it is probable that this process has been hastened in these thyroidless larvae. In the second spring the testis had developed still further in our thyroidless larvae, and in the following July it appeared to be mature (fig. 109). It was joined to the kidney by the usual efferent ducts, in which spermatozoa were found. The testis becomes connected with the kidney in these larvae probably during the first autumn or winter.

In the larger 1918 thyroidless larvae killed in July spermatogenesis was less advanced than in the larger but older 1917 larvae killed in October; hence the rapid growth of the 1918 thyroidless larvae did not accelerate the rate of differentiation of the testes as much as that of the ovaries, although it affected it to some degree.

From the condition of the testes in the oldest thyroidless larvae it might be argued that experimental neoteny, as Allen ('18) has termed it, has been produced. The term neoteny, however, has always implied the ability to reproduce, and objection may be raised to its use here since the females did not reach complete sexual maturity and reproduction by thyroidless larvae has not actually occurred.

#### SUMMARY AND CONCLUSIONS

The thyroid anlage may be removed from young amphibian embryos before it has begun to differentiate. The thyroidless larvae in which muscular defects are not produced by the operation grow more rapidly than the controls and are often twice as large in volume as the normal larvae at the time when the latter reach their maximum size. Ultimately they may become more than three times as large as the controls. The submaxillary muscles are especially liable to injury by the operation and larvae so injured grow less rapidly than the controls, since they suffer from inanition, owing to difficulty in eating.

Thyroidless larvae do not metamorphose even when kept alive for a year after the metamorphosis of the controls, and hence are probably unable ever to metamorphose at all.

Failure in metamorphosis is attributed to faulty metabolism, especially of calcium, since one of the most striking effects of thyroid removal is a deficiency in calcification and ossification of the skeleton.

Older thyroidless larvae will live for a time out of water, if kept in a moist chamber. They decrease rapidly in volume and their tails shorten considerably, but the skeleton does not grow.

Thyroidless larvae are able to breathe with their lungs, which develop normally.

Thyroidless larvae retain the power of regeneration of lost parts to a limited extent for more than a year at least.

The brain grows more slowly in thyroidless than in normal larvae, both actually and relatively. It differentiates slowly and becomes ultimately much larger than the fully differentiated normal brain, while still only partially differentiated. In thyroidless larvae kept alive a year the brain compared with young frogs becomes fully differentiated in shape, excepting the anterior end of the hemispheres. The brain of the normal animal increases in relative size during metamorphosis.

The liver of normal animals changes in shape during metamorphosis and decreases in size. In thyroidless larvae the liver becomes relatively very large and tends to assume the mature shape, but never quite differentiates because a coil of gut is half embedded in it.

The hypophysis undergoes hyperplasia after removal of the thyroid, but is unable to compensate entirely for the loss of the latter. This hyperplasia may account for the rapid growth of thyroidless larvae. The hypophysis normally increases in relative size during metamorphosis.

The thymus persists in older thyroidless larvae. In thyroidless and control larvae of the same size the thymus glands are of the same size. The thymus is relatively larger in young frogs than in larvae, since it decreases less during metamorphosis than does the body as a whole. In larger thyroidless larvae the

thymus is relatively larger than in smaller thyroidless or control larvae.

The epithelioid (parathyroid) bodies are not affected by thyroidectomy until after metamorphosis of the controls. They become relatively large in large thyroidless larvae. They increase in relative size during metamorphosis.

The thyroid is necessary for metamorphosis of frog larvae kept on normal diet. The thyroid of normal animals increases in relative size during metamorphosis, but shows no actual change. The thyroid is necessary for full ossification of the skeleton, but may not be necessary for the atrophy of tissues that occurs during metamorphosis. Excessive amounts of thyroid hasten metamorphosis, but a thyroid much smaller than normal is sufficient to permit metamorphosis.

The kidneys become relatively large in thyroidless larvae. During metamorphosis in normal animals the kidneys decrease in size, but less than does the body as a whole.

The spleen does not appear to be directly affected by thyroidectomy, but it becomes large in large larvae. The variability in the size of the spleen is very great. The spleen does not appear to change in actual size during metamorphosis, but increases in relative size.

The internal gills persist in animals kept in the larval condition by thyroidectomy. The lungs develop and become functional in both normal and thyroidless larvae.

The intestine becomes long in large thyroidless larvae and retains its larval character.

The ovaries become large in thyroidless larvae, and oocytes develop in them. The growth and differentiation of the ovary are to a limited extent independent of the growth and differentiation of the body as a whole. The oviducts do not develop, at least during the first year and a quarter. Maturation did not occur in the ovaries of thyroidless larvae in our experiments. The ovaries of normal animals are not directly affected by metamorphosis.

The testes become fully mature in thyroidless larvae, producing spermatozoa which escape into the kidneys. Young frogs have

relatively larger testes than do normal larvae. Synapsis in the testis is hastened in point of time by thyroidectomy.

The condition in thyroidless larvae cannot be called experimental neoteny, according to the present definition of the term. The females do not produce mature ova nor develop oviducts, and hence the larvae cannot reproduce.

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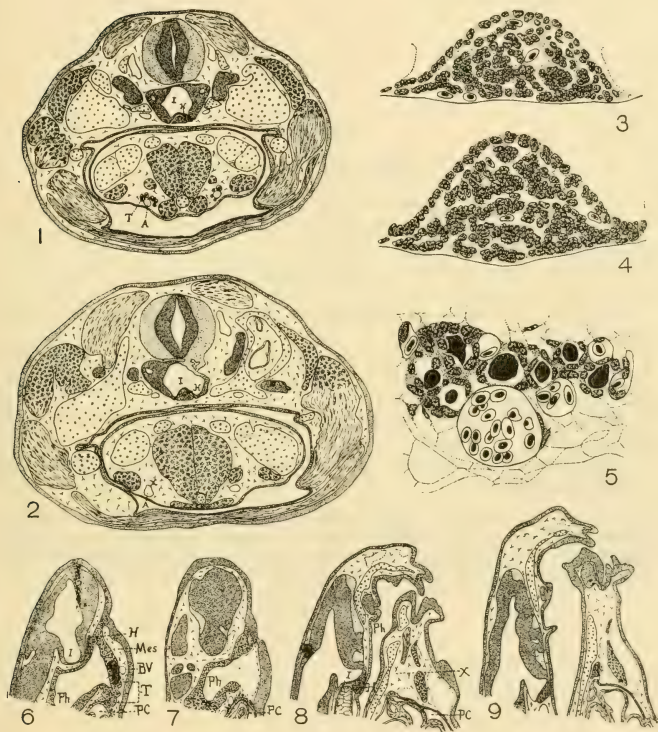
## PLATES

## PLATE 1

### EXPLANATION OF FIGURES

- 1 *Amblystoma*. Control. Tot., 24 mm. Transverse section. 5/15 to 8/15/16.  $\times 9$
- 2 *Amblystoma*. Thyroidless. Tot., 30 mm. Transverse section. 5/15 to 8/15/16.  $\times 9$
- 3 *Amblystoma*. Control. Tot., 24 mm. Hypophysis.  $\times 96$ .
- 4 *Amblystoma*. Thyroidless. Tot., 30 mm. Hypophysis.  $\times 96$ .
- 5 *Amblystoma*. Control. Tot., 24 mm. Thyroid.  $\times 96$ .
- 6 *R. sylvatica*. Control. Tot., 6.5 mm. 4/20/16.  $\times 20$ .
- 7 *R. sylvatica*. Thyroidless. Tot., 6.5 mm. One hour after thyroidectomy.  $\times 20$ .
- 8 *R. sylvatica*. Thyroidless. Tot., 10.5 mm. Four days after thyroidectomy.  $\times 20$ .
- 9 *R. sylvatica*. Control. Tot., 10 mm.  $\times 20$ .

In stating the length of the animals, *Tot.* = total length. The dates given refer to the date of the beginning of the experiment and the date of killing the animal e.g., 5/15 to 8/15/16 = May 15th to August 15th, 1916. Abbreviations: *A.*, aorta; *B. V.*, blood-vessel; *H.*, hypophysis; *Mes.*, mesoderm; *P.C.*, pericardial cavity; *Ph.*, pharynx; *T.*, thyroid; *X.*, location of thyroid in normal larvae.





## PLATE 2

### EXPLANATION OF FIGURES

*R. sylvatica*. All natural size. Drawn in 70 per cent alcohol.

10 and 11 Thyroidless. 4/28/17 to 7/10/18. Alive: Tot., 72 mm., B., 25 mm.; fixed: Tot., 69 mm., B., 25 mm.

12 and 13 Control. 4/14 to 5/29/18. Alive: Tot., 47 mm., B., 18.5 mm.; fixed: Tot., 43 mm., B., 18 mm.

14 and 15 Thyroidless. 4/14 to 5/28/18. Alive: Tot., 50 mm., B., 19 mm.; fixed: Tot., 46 mm., B., 18 mm.

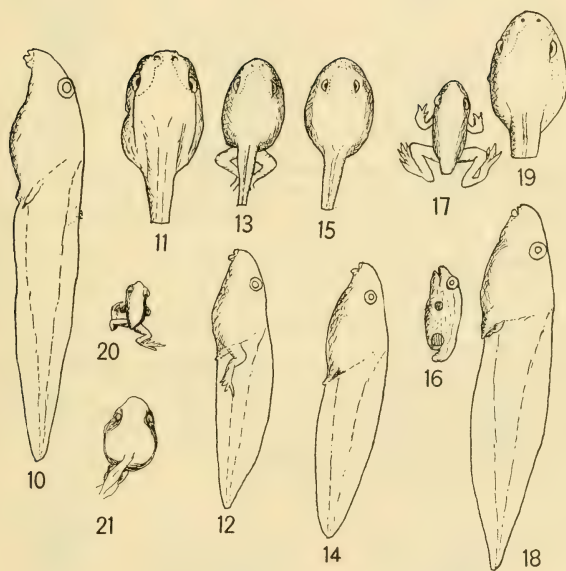
16 and 17 Control. 4/14 to 6/1/18. Alive: B., 15 mm.; fixed. B., 14 mm.;

18 and 19 Thyroidless. 4/14 to 7/13/18. Alive: Tot., 65 mm., B., 25 mm.; fixed: Tot., 60 mm., B., 23 mm.

20 Precocious frog with regenerated thyroid. 4/11 to 7/10/17. Alive: B., 10.5 mm.; fixed: B., 8 mm.

21 Control. 4/11 to 7/10/17. Alive: Tot., 40 mm., B., 15.5 mm.; fixed: Tot., 37 mm., B., 14 mm.

The dates given refer to the beginning of the experiment and the date of killing the animal, thus indicating the age of each specimen. In stating the length, Tot. = total length; B., = nose-anus length. These specimens were selected as typical of the different groups. For the full number see table 1.



### PLATE 3

#### EXPLANATION OF FIGURES

*R. sylvatica*. Brains after fixation. Representative specimens. Note particularly the shape of the optic lobes and fore-brain.

22 Control larva. 5/3 to 5/23/18. Alive: Tot., 36 mm., B., 15.5 mm.; fixed: Tot., 34 mm., B., 13 mm.

23 Control frog. 4/14 to 6/5/18. Alive: B., 15 mm.; fixed: B., 14 mm.

24 Control larva. 4/14 to 5/28/18. Alive: Tot., 48 mm., B., 20 mm.; fixed: Tot., 45 mm., B., 19 mm.

25 Thyroidless larva. 4/14 to 5/20/18. Alive: Tot., 50 mm., B., 20 mm.; fixed: Tot., 47 mm., B., 19 mm.

26 Thyroidless larva. 4/15 to 10/13/17. Alive: Tot., 68 mm., B., 25 mm.; fixed: Tot., 60 mm., B., 23 mm.

27 Thyroidless larva. 4/28/17 to 5/7/18. Alive: Tot., 60 mm., B., 22 mm.; fixed: Tot., 52 mm., B., 21 mm.

Abbreviations and dates as in plate 2.



22



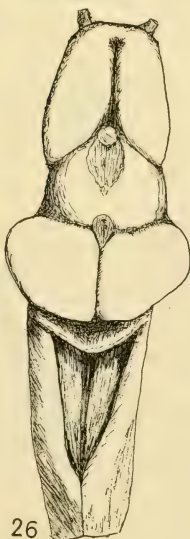
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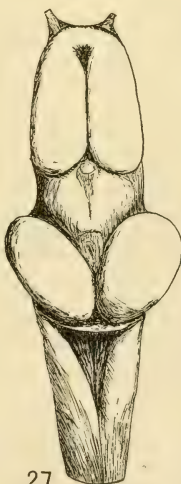
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27



## PLATE 4

### EXPLANATION OF FIGURES

*R. sylvatica*. Organs after fixation. Representative camera-lucida drawings selected from drawings of organs from sixty-nine complete and forty partial autopsies.

#### Heart, $\times 5.1$ .

28 Thyroidless larvae. 5/2 to 5/20/18. Alive: Tot., 50 mm., B., 20 mm.; fixed: Tot., 47 mm., B., 19 mm.

29 Control larva. 4/14 to 5/28/18. Alive: Tot., 48 mm., B., 20 mm.; fixed: Tot., 45 mm., B., 19 mm.

30 Control frog. 4/14 to 6/5/18. Alive: Tot., 16 mm., B., 15 mm.; fixed: Tot., 15 mm., B., 14 mm.

31 Thyroidless larva. 5/3 to 10/17/17. Alive: Tot., 58 mm., B., 22 mm.; fixed: Tot., 50 mm., B., 20 mm.

#### Liver, $\times 5.1$ . Ventral view

32 Thyroidless larva (same as fig. 28).

33 Control larva (same as fig. 29).

34 Control frog (same as fig. 30).

35 Thyroidless larva. 4/14 to 10/5/17. Alive: Tot., 65 mm., B., 25 mm.; fixed: Tot., 61 mm., B., 21 mm.

#### Hypophysis, $\times 20.5$ . Parallel ventral view and transverse section.

36 and 37 Thyroidless larva. 5/2 to 6/8/18. Alive: Tot., 45 mm., B., 18 mm.; fixed: Tot., 42 mm., B., 17.5 mm. A = anterior, S = superior, and I = inferior lobes.

38 and 39 Control larva. 4/30 to 6/4/18. Alive: Tot., 47 mm., B., 18 mm.; fixed: Tot., 45 mm., B., 17.5 mm.

40 and 41 Control frog. 4/14 to 6/4/18. Alive: Tot., 16 mm., B., 14 mm.; fixed: Tot., 15 mm., B., 13 mm. The anterior lobe may be in the form of two separate lobes.

42 and 43 Thyroidless larva. 4/22 to 5/30/18. Alive: Tot., 55 mm., B., 22 mm.; fixed: Tot., 53 mm., B., 21 mm.

44 and 45 Thyroidless larva. 4/12/17 to 7/5/18. Alive: Tot., 72 mm., B., 25 mm.; fixed: Tot., 68 mm., B., 24.5 mm.

Thymus,  $\times 28$ . Two views of each gland to show the three dimensions.

46 A and B Thyroidless larva. 5/2 to 6/1/18. Alive: Tot., 41 mm., B., 18 mm.; fixed: Tot., 41 mm., B., 17 mm.

47 A and B Control larva. 4/30 to 6/4/18. Alive: Tot., 43 mm., B., 18.; fixed: Tot., 40 mm., B., 17 mm.

48 A and B Control frog (same as fig. 40).

49 A and B Thyroidless larva (same as fig. 35).

Epithelioid (parathyroid) bodies,  $\times 40.8$ . Ventral view of the two pairs from each animal

50 Control larva (same as fig. 29).

51 Thyroidless larva (same as fig. 28).

52 Control frog (same as fig. 30).

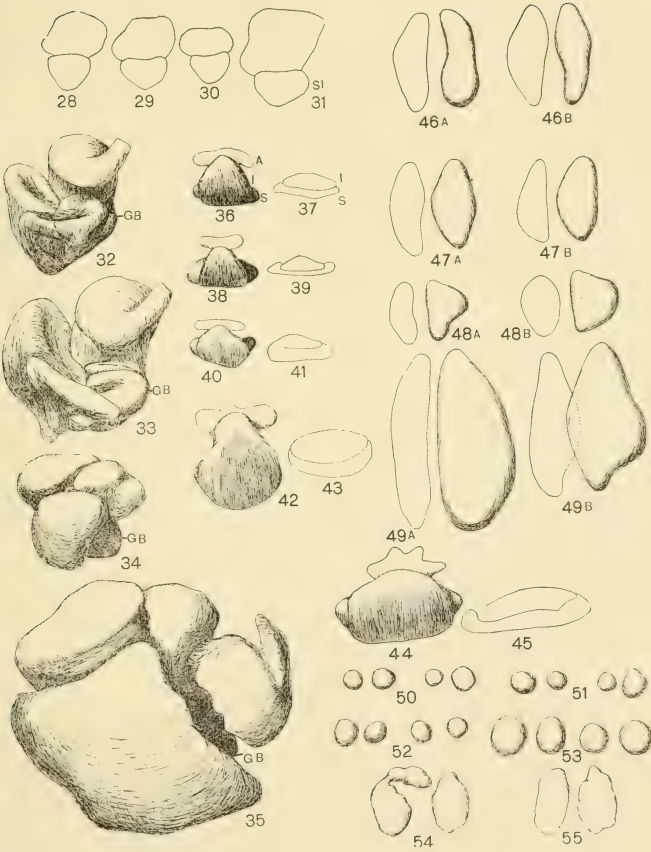
53 Thyroidless larva (same as fig. 42).

#### Thyroid, $\times 20.5$ . Ventral view of the pair in each animal

54 Control larva (same as fig. 29).

55 Control frog (same as fig. 30).

G. B. = gall bladder. Other abbreviations and dates as in plate 2. The original drawings are three times as large as these.

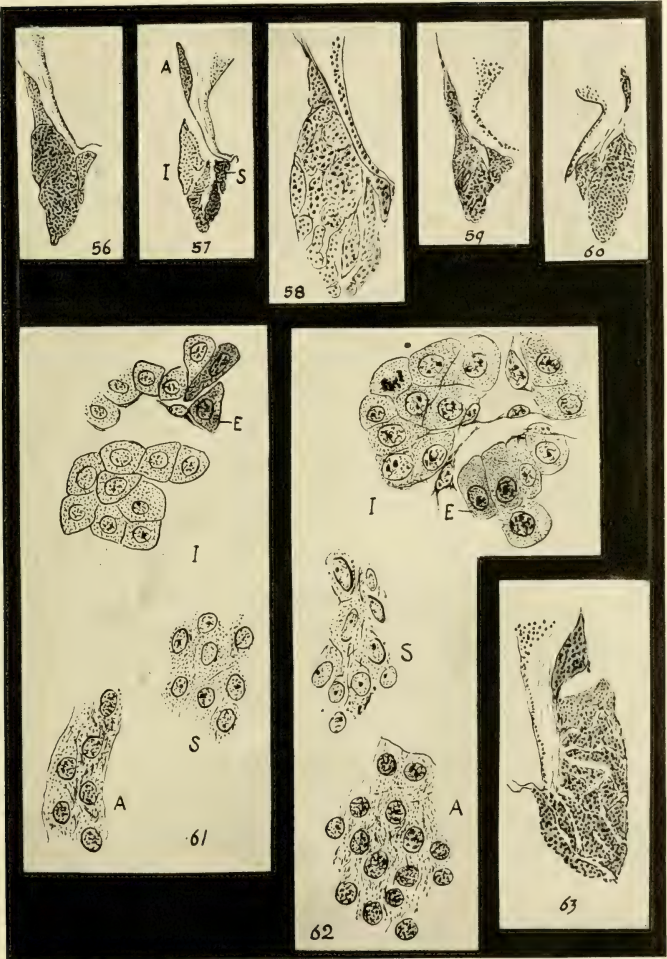


## PLATE 5

### EXPLANATION OF FIGURES

*R. sylvatica*. Hypophysis. Representative specimens. Abbreviations: *A.*, anterior, *S.*, superior, and *I.*, inferior lobes of hypophysis; *E.*, eosinophilic cells of inferior lobe; *In.*, infundibulum. Median sagittal sections.

- 56 Normal half-grown larva (same as fig. 21).  $\times 65$ .
- 57 Full-grown normal larva before metamorphosis.  $\times 65$ .
- 58 Thyroidless larva of same age as that of figure 57, but slightly larger. Shows hyperplasia.  $\times 65$ .
- 59 Young frog.  $\times 65$ .
- 60 Young frog. Comparison with figure 59 shows variation in size of hypophysis.  $\times 65$ .
- 61 Cells from the three lobes shown in figure 57.  $\times 670$ .
- 62 Cells from figure 58.  $\times 670$ .
- 63 Large thyroidless larva five months older than larva of figure 58.  $\times 65$ .





## PLATE 6

### EXPLANATION OF FIGURES

#### *R. sylvatica*. Various organs

- 64 Thymus of full-grown larva.  $\times 40$ .
- 65 Thymus of thyroidless larva larger, than that of figure 64, but same age.  
 $\times 40$ .
- 66 Small lymphocytes and large thymic cells from thymus shown in figure  
64.  $\times 670$ .
- 67 From thymus shown in figure 65.  $\times 670$ .
- 68 Epithelioid body from same larva as figure 64. *Cap.*, capsule.  $\times 670$ .
- 69 Epithelioid body from larva of figure 65.  $\times 670$ .
- 70 Small regenerated thyroid, showing unusual structure.  $\times 160$ .
- 71 Normal thyroid of young frog. The structure and size are the same as  
before metamorphosis.  $\times 160$ .
- 72 From figure 70.  $\times 670$ .
- 73 From figure 71.  $\times 670$ .
- 74 Thyroid of half-grown normal larva.  $\times 160$ .
- 75 Thyroid of precocious frog (fig. 20). This thyroid regenerated and hy-  
pertrophied. Compare with figure 74 from a normal larva of the same age.  
 $\times 160$ .
- 76 Spleen of small normal larva (23 mm.) showing the two types of splenic  
cells.  $\times 670$ .
- 77 Spleen of control larva at maximum length before metamorphosis.  $\times$   
670.
- 78 Spleen of thyroidless larva of same age as that of figure 77.  $\times 670$ .



## PLATE 7

### EXPLANATION OF FIGURES

*R. sylvatica*. Representative specimens from sixty-nine complete and forty partial autopsies. All removed organs were drawn with a camera lucida.

Abbreviations: *Ov.*, ovary; *T.*, testis; *S.*, spleen; *K.*, kidney; *F. B.*, fat body. Other abbreviations and dates as in plate 2.

#### Ovaries, kidneys, and spleen. $\times 8$

79 Control larvae. 4/14 to 6/2/18. Alive: Tot., 45 mm., B., 18.5 mm.; fixed: Tot., 42 mm., B., 17.5 mm.

80 Thyroidless larva. 5/2 to 6/8/18. Alive: Tot., 45 mm.; fixed: Tot., 42 mm., B., 17.5 mm.

81 Control frog. 4/14 to 6/5/18. Alive: Tot., 15 mm., B., 14 mm.; fixed: Tot., 14 mm., B., 13 mm.

82 Thyroidless larva. Alive: Tot., 53 mm., B., 22 mm.; fixed: Tot., 53 mm., B., 21 mm.

83 Thyroidless larva. 5/3 to 10/17/17. Alive: Tot., 65 mm., B., 22 mm.; fixed: Tot., 60 mm., B., 21 mm.

84 Thyroidless larva. 4/28 to 12/21/17. Alive: Tot., 54 mm., B., 22 mm.; fixed: Tot., 50 mm., B., 21 mm.

85 Thyroidless larva. 4/28/17 to 7/10/18. Alive: Tot., 68 mm., B., 22 mm.; fixed: Tot., 56 mm., B., 22 mm.

86 Control frog. 4/7 to 8/12/17. Alive: Tot., 14.5 mm., B., 14 mm.; fixed: Tot., 14 mm., B., 13.5 mm.

87 Thyroidless larva. 4/14 to 7/13/18. Alive: Tot., 65 mm., B., 24 mm.; fixed: Tot., 60 mm., B., 23 mm.

#### Testes. $\times 8$

88 Thyroidless larva. 5/2 to 6/8/18. Alive: Tot., 45 mm., B., 18 mm.; fixed: Tot., 42 mm., B., 17.5 mm.

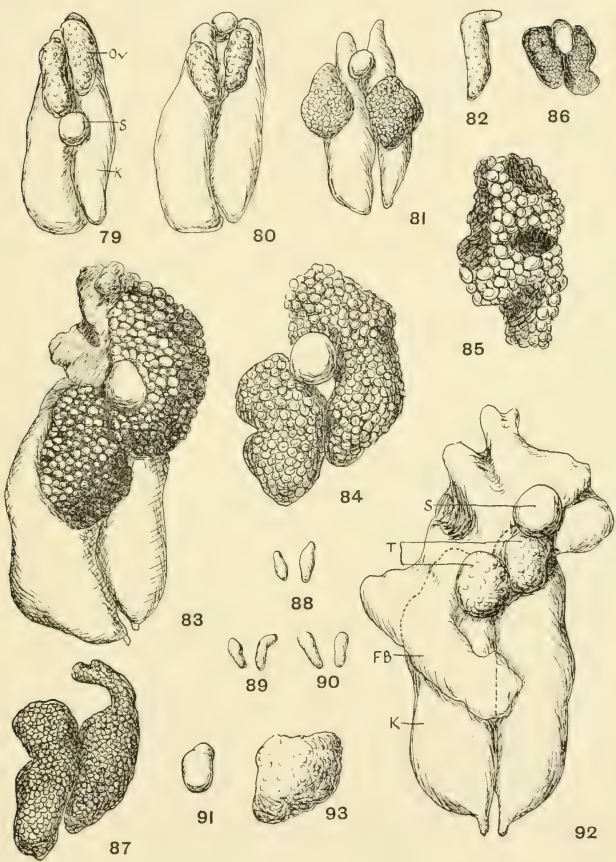
89 Control larva. 4/18 to 5/28/18. Alive: Tot., 45 mm., B., 18.5 mm.; fixed: Tot., 42.5 mm., B., 18 mm.

90 Control frog. 4/14 to 6/5/18. Alive, 15 mm., fixed, 14 mm.

91 Thyroidless larva. 4/14 to 7/15/18. Alive: Tot., 54 mm., B., 20 mm.; fixed: Tot., 48.5 mm., B., 17.5 mm.

92 Thyroidless larva. 5/3 to 10/17/17. Alive: Tot., 68 mm., B., 23 mm.; fixed: Tot., 63 mm., B., 21 mm.

93 Thyroidless larva. 4/28/17 to 7/6/18. Alive: Tot., 72 mm., B., 25 mm.; fixed: Tot., 68.5 mm., B., 24.5 mm.



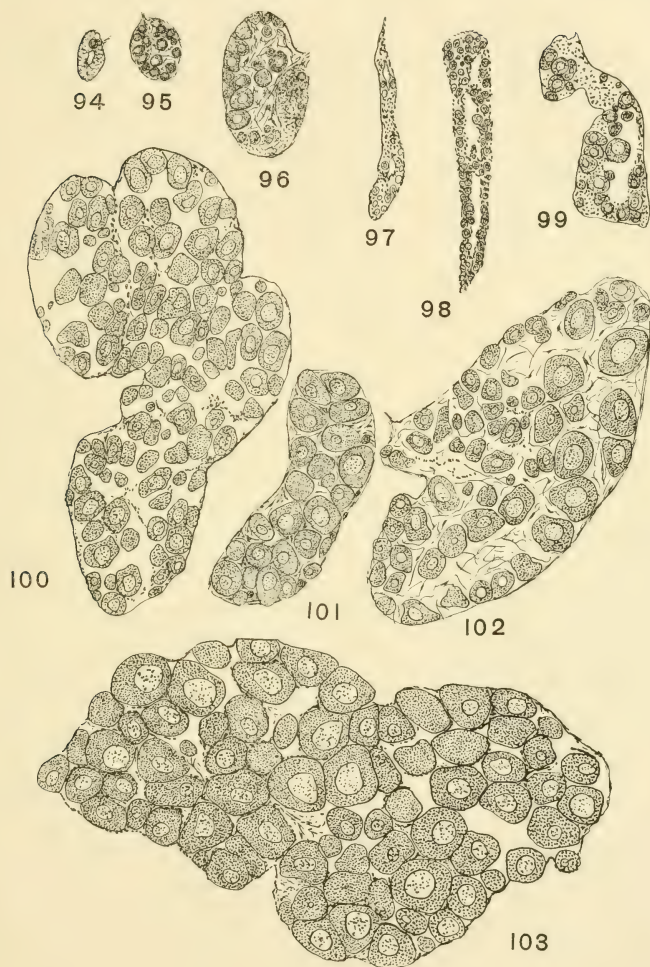


## PLATE 8

### EXPLANATION OF FIGURES

*R. sylvatica*. Ovaries,  $\times 36$

- 94 Half-grown normal larva (fig. 21). Oocytes,  $50\ \mu$ .  
95 Precocious frog (fig. 20). Oocytes,  $75\ \mu$ .  
96 Young normal frog (1917). Oocytes,  $120\ \mu$ .  
97 Young thyroidless larva (1918). Large as control larva at metamorphosis. Oocytes,  $50\ \mu$  (smaller oocytes than those of 1917 larvae of same size).  
98 Thyroidless larva (1918) (5 weeks old). Much younger than that of figure 97, but of much larger size, oocytes ( $60\ \mu$ ) are relatively of the same size.  
99 Thyroidless larva one-half the size of larva of figure 98, but more than twice as old (eleven weeks) Oocytes ( $130\ \mu$ ) are relatively about four times as large in diameter as those in figure 98.  
100 Thyroidless larva (1918). Very little older than larva of figure 99 (twelve weeks), but nearly four times as large. Oocytes ( $180\ \mu$ ) are relatively smaller than those of figure 99.  
101 Thyroidless larva (6 months old), killed in October, 1917. Oocytes,  $200\ \mu$ .  
102 Thyroidless larva killed December 21, 1917 (eight months old). Oocytes,  $250\ \mu$ .  
103 Thyroidless larva fifteen months after thyroidectomy. Oocytes,  $300\ \mu$ . Measurements represent the diameter of the largest oocytes.



## PLATE 9

### EXPLANATION OF FIGURES

#### *R. sylvatica*. Testes

104 A, T. S. Control larva at beginning of metamorphosis (July 29, 1917).  
× 34. B, same, showing beginning of tubule formation, but no synapsis. × 240.

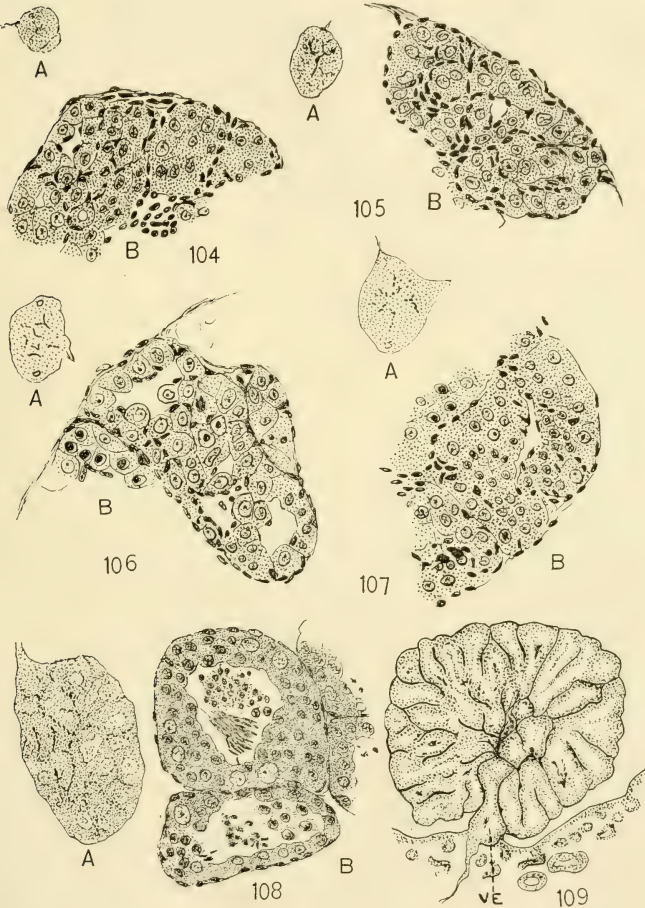
105 A, T. S. Young control frog. × 34. B, same. × 240.

106 A, T. S. Thyroidless larva larger but of same age as larva of figure  
104. × 34. B, same, showing advance in tubule formation, but no synapsis.  
× 240.

107 A, T. S. Large thyroidless larva, killed four months after thyroidectomy  
(August 20, 1917). × 34. B, same, spermatogenesis begun. × 240.

108 A, T. S. Large thyroidless larva, six months after thyroidectomy. × 34.  
B, same, showing spermatozoa in tubules. × 240.

109 Large thyroidless larva one year older than that shown in figure 106.  
Shows fully matured testis. *V. E.*, efferent tubule leading into kidney and con-  
taining spermatozoa. × 34.





Resumen por la autora, Helen Dean King.

Estudios sobre "inbreeding."

IV. Nuevos estudios sobre los efectos de "inbreeding" sobre el crecimiento y variabilidad de peso de la rata albina.

Los datos publicados en el presente trabajo demuestran el crecimiento y la variabilidad de peso de más de 600 ratas albinas pertenecientes a las generaciones comprendidas entre la 16 y 25 generación de un tronco "inbred" hermano con hermana, pertenecientes ambos a la misma cría. Los principales puntos de interés son los siguientes: 1. El "inbreeding" continuo no ha producido efecto perjudicial alguno en el tronco albino original en lo referente a la marcha y extensión del aumento de peso del cuerpo, ni tampoco ha alterado la forma de la gráfica de crecimiento de los dos sexos. 2. Las relaciones normales de peso de los sexos no se han alterado después de 25 generaciones de "inbreeding." 3. La variabilidad de los pesos de estos animales es relativamente alta en todas las edades y no decrece cuando el "inbreeding" avanza. 4. Una comparación de la variabilidad de los pesos de diferentes series de albinos del mismo tronco con los de las ratas "inbred" indica que el aumento de variabilidad en las últimas se debe a la acción del medio ambiente y a la acción de la nutrición, no al "inbreeding."

Translation by José F. Nonidez  
Carnegie Institution of Washington

## STUDIES ON INBREEDING

### IV. A FURTHER STUDY OF THE EFFECTS OF INBREEDING ON THE GROWTH AND VARIABILITY IN THE BODY WEIGHT OF THE ALBINO RAT

HELEN DEAN KING

*The Wistar Institute of Anatomy and Biology*

EIGHT CHARTS

In order to complete the series of records for the first twenty-five generations of inbred albino rats, data showing the growth and variability in the body weights of individuals belonging in the sixteenth to the twenty-fifth generations are given in the present paper.

Five litters from each generation of the two inbred series (A and B), comprising a total of 296 males and 310 females, were used for this study. The rats in these litters were selected in the same manner, and they were weighed at the same age periods, as were the individuals of the seventh to the fifteenth generations for which body-weight records were taken (King, '18). The data for the animals in the different generations of the inbred strain are therefore strictly comparable.

During the past three years, when most of the weighings were taken, it was not possible to rear the animals under environmental and nutritive conditions that were as favorable to growth and to fertility as those existing previously. Owing to economic conditions incident to the war, it became necessary to make a radical change in the character of the food that the rats received. The 'scrap' food (carefully sorted table refuse), on which the animals of the earlier generations seemed to thrive exceedingly well, had to be replaced by a ration that consisted, for the most part, of oats and corn, with the occasional addition of various kinds of vegetables and a little meat. Some of the available

substitutes that from time to time were added to the diet in order to vary it, such as alfalfa, linseed and cottonseed meal, proved very injurious to the rats and very materially affected their growth and fertility. For some time, therefore, the food given the animals has been largely in the nature of an experiment, and it has not even yet been possible to work out a ration that produces as rapid and vigorous growth and that is as favorable to reproduction as was the 'scrap' food given previously.

Extremes of temperature, either of heat or of cold, have a very marked effect on the body growth of the rat, as they have on that of mice (Sumner, '09), and many of the animals in the later generations of the inbred strain suffered considerably from this cause. During the excessive cold of the winter of 1917-1918 it was impossible to keep the colony house above the freezing point for days at a time, and in consequence the rats ceased growing at a normal rate and many of them developed pneumonia. The periods of intense heat experienced during the summer of 1918 also had a very deleterious effect on the vitality and on the body growth of the rats. As a result of the combined action of these various factors, all inimical to growth as well as to reproduction, the rats of the eighteenth to the twenty-fifth generations were severely handicapped, and they did not increase in body weight as rapidly, nor did they attain as great a maximum body weight, as did the individuals of the earlier generations. That this decrease in the size of the inbred animals was caused by unfavorable conditions of environment and of nutrition, and not by continued inbreeding, is shown conclusively by the fact that the body weights of hundreds of rats in the outbred-stock colony were just as seriously affected by these adverse conditions as were those of the inbred rats, as will be shown later.

Data showing the average body weights at different ages of 179 males and of 130 females belonging in the sixteenth to the twenty-fifth generations of the A series of inbred rats are given in table 1 and in table 2; similar data for 117 males and for 180 females belonging in the same generations of the B series of inbreds are given in table 3 and in table 4.

TABLE 1

*Showing, by generations, the average body weights at different ages of 179 males belonging in the sixteenth to the twenty-fifth generations of the A series of inbred rats*

AGE	GENERATIONS									
	16	17	18	19	20	21	22	23	24	25
<i>days</i>										
13	19	18	19	18	17	20	19	17	16	18
30	44	44	49	41	42	45	46	44	39	43
60	131	121	135	97	118	115	111	96	83	104
90	188	186	192	142	186	164	165	126	121	163
120	232	228	223	197	237	211	203	169	159	200
151	255	253	259	232	259	244	226	207	188	233
182	274	268	286	262	280	271	250	231	216	252
212	296	277	309	286	289	295	276	243	231	277
243	302	288	328	298	297	311	291	254	246	279
273	321	310	352	298	308	310	308	264	265	292
304	317	321	356	301	309	305	305	279	272	303
334	327	322	365	305	313	311	313	290	276	316
365	333	319	378	308	324	322	320	288	284	325
395	336	332	394	304	334	318	306	298	287	327
425	331	339	376	295	340	319	293	297	290	332
455	320	332	361	295	357	316	296	289	293	322
Number rats weighed	17	17	14	17	15	16	20	21	21	21

TABLE 2

*Showing, by generations, the average body weights at different ages of 130 females belonging in the sixteenth to the twenty-fifth generations of the A series of inbred rats*

AGE	GENERATIONS									
	16	17	18	19	20	21	22	23	24	25
<i>days</i>										
13	19	17	19	17	16	19	17	16	16	17
30	41	44	48	39	40	44	43	42	38	41
60	99	105	110	94	97	95	99	81	79	94
90	141	163	148	134	153	135	142	107	109	131
120	170	178	179	163	177	166	170	135	137	153
151	187	197	195	182	195	192	179	163	160	177
182	206	211	208	197	205	203	183	184	178	180
212	210	214	219	205	215	209	202	190	185	191
243	214	225	222	212	221	213	207	194	187	194
273	230	221	224	214	222	218	208	197	190	204
304	225	233	224	221	216	230	206	206	199	212
334	229	232	220	225	215	236	215	209	189	215
365	240	231	221	223	214	242	217	211	194	223
395	242	227	224	219	210	239	217	209	195	234
425	235	231	224	214	216	231	216	208	190	240
455	243	236	223	216	212	229	215	204	186	230
Number rats weighed	13	11	12	13	13	14	14	13	13	14

Tables 1 to 4 are inserted mainly for reference, but a comparison of the data for the males and females in the various generations brings out clearly the relation between the two sexes as regards their relative body weights at different age periods. In some few instances the average body weights of the males and of the females in a given generation were the same when the animals were thirteen or thirty days old, but after this age the males were the heavier at each period for which records were taken. A similar relation between the body weights of the sexes was also noted for the inbred animals of the seventh to the fifteenth generations (King, '18; tables 1 to 4). Investigations in which large series of stock Albinos were weighed at stated periods (Donaldson, '06; Jackson, '13; King, '15; Hoskins, '16) have shown likewise that, with few exceptions, the average body weight of the males exceeds that of the females at each weighing period. Since the data for all generations of the inbred strain is in full accord with that for various series of stock Albinos, it is evident that inbreeding through twenty-five generations of brother and sister matings has not changed the normal relative body weights of the sexes at any age period for which records have been taken.

For the purpose of analysis and to facilitate a comparison between the growth in body weight of the individuals in the later generations of the inbred series with those in the earlier generations, the body-weight data for the animals belonging in the sixteenth to the twenty-fourth generations of each inbred series were combined in groups of three generations each: the data thus combined are shown in tables 5 to 7. In each of these tables the data for the individuals of the twenty-fifth generation are given separately in order to show the status of the animals at the end of this period of inbreeding.

Data indicating the growth in body weight of males and of females belonging in the various generation groups of the A series of inbreds are shown in table 5.

As a graphic representation of series of data greatly facilitates their comparison, the body-weight data for various groups of albino rats, given in tables 5 to 11, have formed the basis



TABLE 3

*Showing, by generations, the average body weights at different ages of 117 males belonging in the sixteenth to the twenty-fifth generations of the B series of inbred rats*

AGE	GENERATIONS									
	16	17	18	19	20	21	22	23	24	25
<i>days</i>										
13	19	17	20	21	18	19	20	19	18	18
30	52	44	51	48	41	43	48	48	42	42
60	119	111	119	123	109	111	96	114	95	114
90	178	165	181	175	161	168	159	154	143	147
120	213	222	225	197	203	220	188	180	182	180
151	237	245	261	227	233	245	229	212	224	221
182	266	266	284	251	263	267	263	235	228	231
212	284	279	297	277	272	277	279	252	240	252
243	289	289	310	304	279	305	284	258	259	279
273	296	297	319	323	285	312	293	265	272	289
304	304	303	323	324	289	317	300	276	271	299
334	310	319	336	337	298	323	301	291	292	312
365	322	317	322	337	295	328	315	290	305	322
395	337	326	311	352	296	322	309	293	303	339
425	334	354	305	364	290	320	305	300	298	352
455	340	299	299	350	279	322	299	297	287	349
Number rats weighed	12	11	11	11	11	12	13	12	12	12

TABLE 4

*Showing, by generations, the average body weight at different ages of 180 females belonging in the sixteenth to the twenty-fifth generations of the B series of inbred rats*

AGE	GENERATIONS									
	16	17	18	19	20	21	22	23	24	25
<i>days</i>										
13	19	16	17	20	17	18	19	19	17	18
30	47	41	48	44	39	41	44	45	41	41
60	102	93	107	101	92	101	85	97	83	93
90	151	125	157	143	133	149	133	133	119	127
120	166	165	179	167	162	192	151	149	143	152
151	189	180	199	183	179	188	177	166	167	166
182	199	194	212	194	198	199	194	177	177	178
212	211	205	214	205	201	200	199	187	185	187
243	215	214	216	212	216	210	199	196	191	191
273	221	226	218	216	220	210	203	196	194	194
304	224	217	217	221	218	211	200	198	196	202
334	225	224	215	225	217	209	207	208	203	204
365	233	224	220	232	214	212	205	208	211	214
395	239	216	216	232	212	210	207	212	201	213
425	243	238	218	233	207	203	203	210	199	220
455	243	253	217	228	201	202	202	211	200	218
Number rats weighed	16	14	18	19	19	18	18	19	19	20

for the construction of the graphs shown in figures 1 to 8. The graphs in figure 1 show the growth in body weight of four generation groups of male rats belonging in the A series of inbreds (data in table 5). In this, as in some of the other figures, the graphs should properly run very close together or overlap in various places. If, however, the graphs had been drawn in this manner, it would be difficult to follow their course, and therefore

TABLE 5

*Showing the average body weights at different ages of inbred rats of the A series, separated into groups according to the generation to which the individuals belonged*

AGE	MALES				FEMALES			
	Genera- tions 16-18	Genera- tions 19-21	Genera- tions 22-24	Genera- tion 25	Genera- tions 16-18	Genera- tions 19-21	Genera- tions 22-24	Genera- tion 25
<i>days</i>								
13	19	18	17	18	18	17	16	17
30	46	43	42	43	44	41	41	41
60	129	110	96	103	105	95	86	94
90	189	163	137	163	150	139	120	131
120	228	214	177	200	175	169	144	153
151	255	244	206	233	192	190	168	172
182	275	271	231	252	208	202	185	180
212	291	290	249	277	214	209	193	191
243	301	302	264	279	220	215	196	194
273	322	306	280	292	225	218	198	204
304	326	305	286	303	227	221	204	212
334	333	310	294	316	227	222	204	215
365	337	317	298	325	232	224	208	223
395	340	316	299	327	251	220	208	234
425	345	313	294	331	229	218	205	240
455	338	316	293	322	231	218	202	230

the space between them has been arbitrarily widened in some places in order to keep the lines distinct.

While the general course of all of the graphs in figure 1 is much the same, their relative position clearly shows the progressive decrease in body weight that has resulted from the action of unfavorable conditions of environment and of nutrition. The rats in the sixteenth to the eighteenth generations were fed, for the most part, on 'scrap' food, and, as graph A in figure 1 shows,

the males of the A series that belonged to these generations were heavier at all ages than were the males in the later generation groups, excepting at the 243-day period. Rats in the nineteenth to the twenty-first generations were not greatly affected by the change in diet, as for some months it was possible to give them 'scrap' food part of the time. The males of this generation

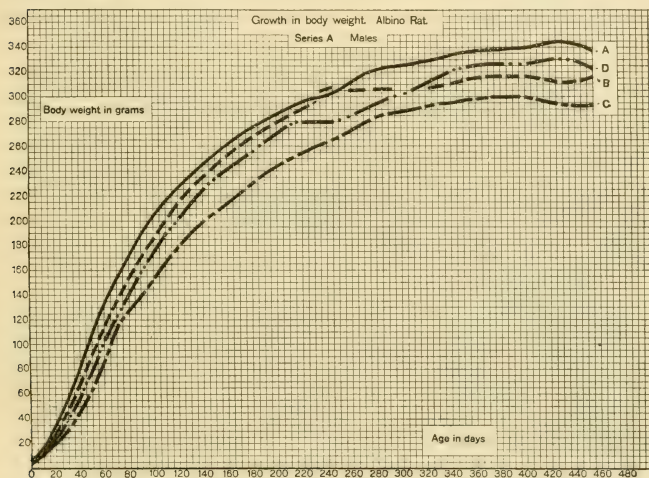


FIG. 1 Graphs showing the increase in the weight of the body with age for males belonging to various generation groups of the A series of inbred rats. A, graph for males of the sixteenth to the eighteenth generations, inclusive; B, graph for males of the nineteenth to the twenty-first generations, inclusive; C, graph for males of the twenty-second to the twenty-fourth generations, inclusive; D, graph for males of the twenty-fifth generation (data in table 5).

group, as the position of graph B indicates, were nearly as large as were those of the earlier generation group during the adolescent period, but in the adult state their body weights fell off rapidly. Individuals in the twenty-second to the twenty-fifth generations of the inbred strain suffered most severely from the altered food conditions as well as from extremes of temperature, and the males of the A series were very inferior in body weight

to those of the preceding generations, as graph C and graph D in figure 1 show. Since the number of weighed individuals in a single generation was comparatively small, it is not surprising that the course of graph D should be rather erratic. At its beginning this graph runs very slightly higher than graph C, but at the 90-day period it begins to rise rapidly, and at 334 days it crosses graph B and subsequently runs above it until the final weighing. In the A series of inbreds the males of the

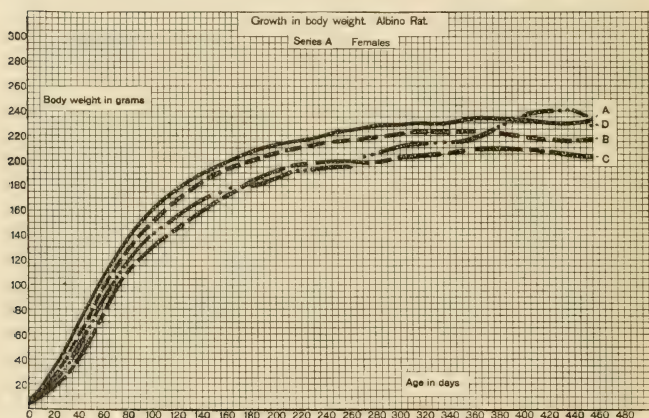


Fig. 2 Graphs showing the increase in the weight of the body with age for females belonging to various generation groups of the A series of inbred rats (data in table 5; lettering as in fig. 1).

twenty-fifth generation were, as a group, superior in body weight to the males of the generation preceding. The superiority of these individuals can be attributed in part to an improvement in the nutritive conditions and in part to the fact that the majority of animals in this generation were born at the time of year that experience has shown is most favorable for body growth in the rat, i.e., the winter months.

Graphs showing the growth in body weight of females belonging to various generation groups of the A series of inbreds are

shown in figure 2. The data from which these graphs were constructed are given in table 5.

In general the relative position of the graphs in figure 2 is much the same as that of the graphs in figure 1. Graph A, representing the body weight increase with age for females of the sixteenth to the eighteenth generations, runs higher than any of the other graphs for the greater part of its course, while the position of the other graphs indicates that there was a gradual decrease in

TABLE 6

*Showing the average body weights at different ages of inbred rats of the B series, separated into groups according to the generation to which the individuals belonged*

AGE	MALES				FEMALES			
	Genera- tions 16-18	Genera- tions 19-21	Genera- tions 22-24	Genera- tion 25	Genera- tions 16-18	Genera- tions 19-21	Genera- tions 22-24	Genera- tion 25
<i>days</i>								
13	19	19	19	18	17	18	18	18
30	49	44	46	43	46	42	43	41
60	116	115	102	114	102	98	89	93
90	175	168	152	147	146	141	128	127
120	220	207	184	180	170	173	148	152
151	249	235	216	211	190	183	170	166
182	271	260	243	231	202	197	183	178
212	286	275	258	252	211	202	190	187
243	295	297	268	279	215	213	195	191
273	303	308	277	289	221	215	198	194
304	309	311	283	299	220	216	198	202
334	319	319	296	312	222	217	206	203
365	320	319	304	322	227	219	207	210
395	326	319	301	339	230	217	207	213
425	328	323	301	352	234	213	204	220
455	321	318	296	349	236	211	204	218

the body growth of the animals as inbreeding advanced. The females of the twenty-fifth generation (graph D) were, on the whole, slightly heavier than were the females of the preceding generation group (graph C).

Table 6 gives data showing the average body weights at different age periods of males and of females belonging to various generation groups of the B series of inbreds.



The data given in table 6 served as the basis of construction for the graphs shown in figure 3 and in figure 4.

A comparison of the graphs in figures 3 and 4 with the corresponding graphs in figures 1 and 2 shows that there was very little difference between the two inbred series (A and B) as regards the body-weight increase with age in the animals of the various generation groups. In the B series, as in the A series, males and

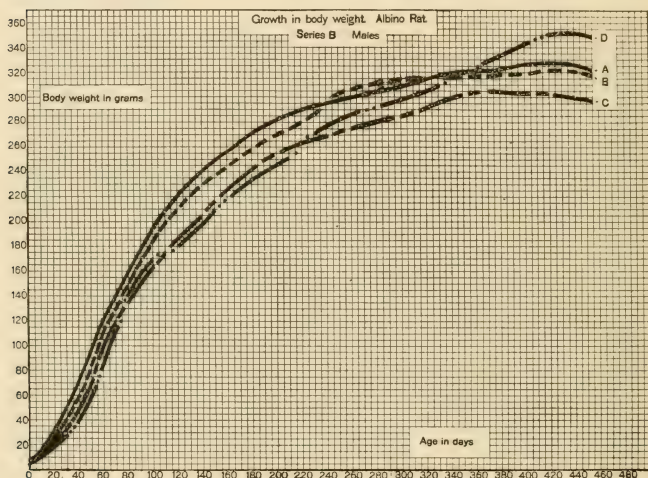


Fig. 3 Graphs showing the increase in the weight of the body with age for males belonging to various generation groups of the B series of inbred rats (data in table 6; lettering as in fig. 1).

females in the sixteenth to the eighteenth generation groups (graph A) were heavier animals at any given age than were those of subsequent generations; while the rats of the twenty-second to the twenty-fourth generation groups showed a much less vigorous growth than did the animals in the earlier groups. The rats in the twenty-fifth generation of the B series increased in body weight very slowly during the adolescent period, as the position of graph D in figures 3 and 4 indicates; but in the adult

state their growth was much more vigorous, and their body weights, especially those of the males, compare favorably with the weights of the animals in the group comprising the rats of the sixteenth to the eighteenth generations (graph A).

An examination of figures 1 to 4 brings out one fact of considerable interest: all of the graphs have the same general form, although they vary somewhat in height. As the form of these graphs is practically the same as that of the growth graphs for

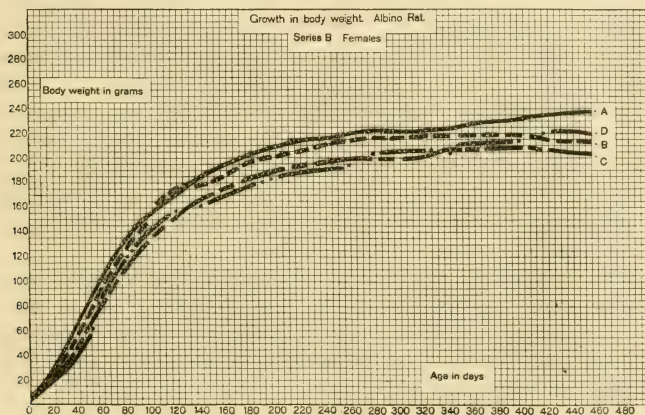


Fig. 4 Graphs showing the increase in the weight of the body with age for females belonging to various generation groups of the B series of inbred rats (data in table 6; lettering as in fig. 1).

stock Albinos as determined by Donaldson ('06) and others, it follows that close inbreeding, continued through many generations, does not alter the character of the growth graph for the albino rat. Theoretically, it might be expected, perhaps, that long-continued inbreeding would cause a slowing up of the growth processes, since the animals totally lack the stimulus to growth that a condition of heterozygosis seems to give in many cases (East and Hayes, '12; Jones, '18). The body weights of the animals in the sixteenth to the twenty-fifth generations of the inbred strain tended to lag somewhat during early postnatal

life (figures 7 and 8, graph B), but this was undoubtedly due to the action of environmental and nutritive conditions, not to inbreeding. Any agency influencing growth, whether it be beneficial or detrimental, naturally produces its greatest effect during the period when growth is normally most rapid and vigorous. Since unfavorable conditions of environment and of nutrition unquestionably limited the extent of body growth in the animals of the later generations of the inbred strain, it is very probable that these factors also lessened the rate of growth during the early life of the individuals. If body growth in the inbred rats of future generations is retarded during the adolescent period, although the environmental and nutritive conditions under which the animals live are such that they produce rapid and vigorous growth in outbred stock Albinos, the change in the rate of growth can be ascribed to the effects of inbreeding. As far as the experiment has gone at present, the evidence does not warrant the conclusion that inbreeding per se has altered the form of the growth graph to any appreciable extent.

The body-weight data for the animals in various generation groups of the two inbred series, as given in table 5 and in table 6, were combined in order to show the weight increase with age in the individuals of the inbred strain as a whole. The combined data are shown in table 7.

The data in table 7 are not presented graphically, since there was such a close agreement between the corresponding records for the various generation groups of the two series that graphs constructed from the combined data would not differ materially from those given for the separate series (figs. 1 to 4).

Table 8 gives data showing the increase in the weight of the body with age for all of the individuals in the sixteenth to the twenty-fifth generations of the A series of inbreds for which growth records were taken; table 9 shows similar data for individuals of the B series.

A comparison of the data in table 8 with corresponding data in table 9 shows that the rats in the two inbred series were much alike as regards the rate and extent of their growth in body weight. To show this similarity more clearly, weight data for the males of the two series are presented graphically in figure 5.

TABLE 7

*Showing the average body weights at different ages of inbred rats of the two series (A, B) separated into groups according to the generation to which the individuals belonged*

AGE	MALES				FEMALES			
	Genera- tions 16-18	Genera- tions 19-21	Genera- tions 22-24	Genera- tion 25	Genera- tions 16-18	Genera- tions 19-21	Genera- tions 22-24	Genera- tion 25
<i>days</i>								
13	19	19	18	18	18	18	17	17
30	47	43	44	43	45	41	42	41
60	124	112	98	107	103	97	88	94
90	183	165	142	157	148	140	125	129
120	225	211	179	193	173	171	146	152
151	253	240	210	225	191	186	169	168
182	274	266	235	244	205	199	184	178
212	289	283	252	268	212	205	192	188
243	298	299	265	279	217	214	196	192
273	313	307	279	291	223	217	198	198
304	318	308	285	301	222	218	200	206
334	326	314	294	314	224	219	205	208
365	328	318	300	324	229	221	208	215
395	312	318	300	332	231	218	207	222
425	336	317	297	339	232	215	205	229
455	329	317	294	332	234	214	203	223

TABLE 8

*Showing the increase in the weight of the body with age for 179 males and for 130 females belonging in the sixteenth to the twenty-fifth generations of the A series of inbred rats*

AGE	MALES				FEMALES			
	Body weight			Number of in- dividuals	Body weight			Number of in- dividuals
	Average	Highest	Lowest.		Average	Highest	Lowest	
<i>days</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>		<i>grams</i>	<i>grams</i>	<i>grams</i>	
13	18	24	14	179	17	24	14	130
30	43	54	36	179	42	57	33	130
60	109	205	58	179	95	158	62	130
90	161	268	92	179	135	187	80	121
120	203	294	128	179	162	218	108	125
151	232	321	163	178	182	235	133	123
182	256	361	196	178	196	238	162	122
212	274	382	192	170	203	246	159	116
243	285	404	199	162	208	268	157	110
273	298	432	215	148	212	268	178	100
304	302	413	213	141	215	279	169	95
334	308	410	213	127	216	273	174	91
365	314	418	223	116	220	283	168	86
395	308	421	231	103	220	305	164	80
425	313	485	227	85	218	298	169	68
455	311	447	223	75	214	269	162	58



The relative position of the graphs in figure 5 shows that during the early growth stages males of the B series of inbreds were slightly heavier at any given age than were the males of the A series; in the period from 100 to 300 days the advantage in body weight was with the males of the A series; beyond this age males of the B series were again the heavier. In the adult state the space between the graphs represents a difference of only about

TABLE 9

*Showing the increase in the weight of the body with age for 117 males and for 180 females belonging in the sixteenth to the twenty-fifth generations of the B series of inbred rats*

AGE	MALES				FEMALES			
	BODY WEIGHT			Number of in- dividuals	BODY WEIGHT			Number of in- dividuals
	Average	Highest	Lowest		Average	Highest	Lowest	
<i>days</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>		<i>grams</i>	<i>grams</i>	<i>grams</i>	
13	19	24	15	117	18	22	14	180
30	46	62	36	117	44	60	34	180
60	111	147	64	117	96	137	63	180
90	163	230	110	117	136	188	98	161
120	201	281	153	117	162	218	122	169
151	230	326	165	117	179	236	136	164
182	255	358	189	116	186	247	143	176
212	270	367	195	115	199	250	157	163
243	285	392	219	113	205	261	169	163
273	294	415	227	106	208	277	168	148
304	300	410	236	104	209	290	172	148
334	311	459	258	96	213	287	181	136
365	315	460	259	85	216	280	180	126
395	317	449	239	78	216	293	177	114
425	319	455	246	64	215	293	171	99
455	315	450	238	56	213	279	168	78

2 per cent in the average body weights of the two groups of animals.

Graphs showing the increase in the weight of the body with age for females of the two inbred series are shown in figure 6. These graphs are based on data given in table 8 and in table 9.

In figure 6, as in figure 5, the graphs lie very close together throughout their entire course. Females in the B series of inbreds were slightly heavier animals than those in the A series



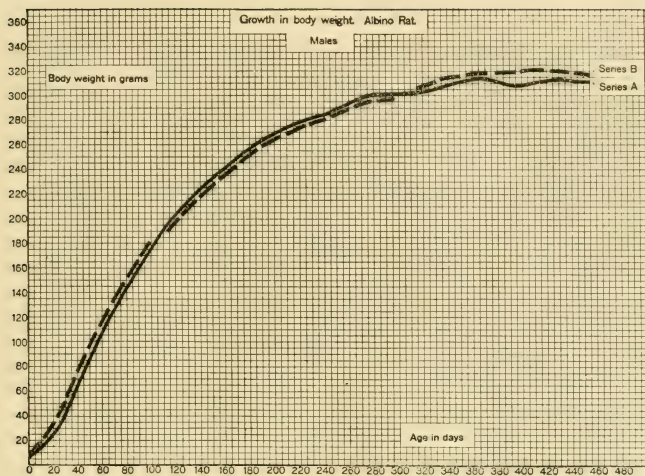


Fig. 5 Graphs showing the increase in the weight of the body with age for males belonging in the sixteenth to the twenty-fifth generations of the two series (A and B) of inbred rats (data in table 8 and in table 9).

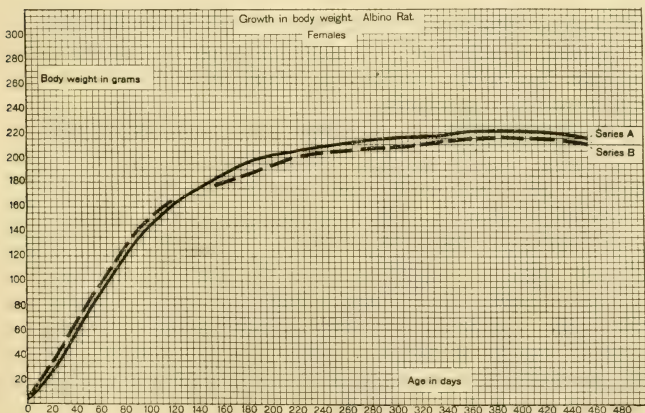


Fig. 6 Graphs showing the increase in the weight of the body with age for females belonging in the sixteenth to the twenty-fifth generations of the two series (A and B) of inbred rats (data in table 8 and in table 9).

during early life, but in the adult state this relation was reversed and the females in the A series were about 2 per cent heavier, as the graphs in figure 6 indicate.

In the seventh to the fifteenth generations of the inbred strain, also, the animals of the two series had about the same average body weight at corresponding age periods, although, as a group, the individuals of the B series were slightly heavier (King, '18; tables 11 and 12). Throughout the period of over nine years that this experiment has been in progress, therefore, body growth in the individuals of the one inbred series has closely paralleled that of the individuals in the other series. If the varying conditions of environment and of nutrition to which the animals of the inbred strain have been subjected have had any influence on the heritable factors on which growth depends, it is evident that they have acted on the animals of both series in a similar way. I am strongly inclined to the opinion that environmental and nutritive conditions do not influence genetic growth factors directly, but that they act by either stimulating or retarding the growth processes.

Body-weight data for a total of 606 individuals, 296 males and 310 females, belonging in the sixteenth to the twenty-fifth generations of the inbred strain are given in table 10. Reference to this table, which is a combination of the data in table 8 and in table 9, will be made later.

In connection with another problem I have recently taken a series of body-weight records for a second group of outbred stock Albinos. Supposedly these rats represented the best stock in our colony at the time that the investigation was begun (1916), as care was taken to select for breeding the largest and apparently the most vigorous individuals from the large number available for this purpose. These stock Albinos were reared simultaneously with, and under the same environmental and nutritive conditions, as the inbred rats of the twenty-first to the twenty-fifth generations. The body-weight data for these animals are given in table 11.

A comparison of the body-weight data for the stock Albinos (table 11) with that for the inbred group (table 10) shows that

the inbred rats, both males and females, were much heavier than the stock rats at every age for which records were taken. Not only were the animals in this stock series very inferior in size to those in the first stock series reared in 1913 to 1915 as controls for the inbred strain (King, '15; table 3), but their average body weights during adult life were no greater than those of the rats in the first six generations of the inbred strain which suffered severely from malnutrition (King, '18; table 3).

TABLE 10

*Showing the increase in the weight of the body with age for 296 males and for 310 females belonging in the sixteenth to the twenty-fifth generations of the inbred rats (Series A and B combined)*

AGE	MALES				FEMALES			
	BODY WEIGHT			Number of in- dividuals	BODY WEIGHT			Number of in- dividuals
	Average	Highest	Lowest		Average	Highest	Lowest	
<i>days</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>		<i>grams</i>	<i>grams</i>	<i>grams</i>	
13	18	24	14	296	18	24	14	310
30	44	62	36	296	43	60	33	310
60	110	205	58	296	95	158	62	310
90	161	268	92	296	136	188	80	282
120	202	294	128	296	162	218	108	294
151	232	326	163	295	180	236	133	287
182	255	361	189	294	187	247	143	298
212	272	382	192	285	201	250	157	279
243	285	404	199	275	206	268	157	273
273	296	432	215	254	210	277	168	248
304	301	413	213	245	211	290	169	240
334	310	459	213	223	214	287	174	227
365	314	460	223	201	218	283	168	212
395	312	449	231	181	218	305	164	194
425	315	485	227	149	216	298	169	167
455	312	450	223	131	213	293	162	136

To facilitate a comparison between the body growth of inbred rats belonging in various generation groups and that of outbred stock Albinos, graphs showing the weight increase with age in two groups of inbred rats and in two groups of stock rats are given in figure 7 and in figure 8.

Growth graphs for various series of male rats are shown in figure 7.

In figure 7, graph A runs considerably above all of the other graphs, except at the thirteen-day period, thus showing that the growth of the males in the seventh to the fifteenth generations of the inbred strain was exceptionally vigorous. Males in the sixteenth to the twenty-fifth generations were relatively small: in the adult state their average body weights were about 9 per

TABLE II

*Showing the increase in the weight of the body with age and the coefficients of variability for 165 males and for 139 females belonging to a series of stock albino rats that were reared under the same environmental and nutritive conditions as the inbred rats belonging in the twenty-first to the twenty-fifth generations*

AGE	MALES			FEMALES		
	Average body weight	Coefficients of variability	Number of individuals	Average body weight	Coefficients of variability	Number of individuals
<i>days</i>	<i>grams</i>			<i>grams</i>		
13	15	15.8±0.92	165	17	16.0±0.84	139
30	40	18.4±1.01	165	39	17.6±1.04	139
60	94	21.3±0.83	150	83	20.2±0.83	131
90	126	20.0±0.97	149	116	17.4±0.75	122
120	173	19.6±0.76	149	137	14.9±0.66	118
151	195	18.1±0.71	149	152	12.0±0.52	120
182	213	15.9±0.63	147	164	11.3±0.51	111
212	226	18.0±0.71	143	171	13.7±0.65	102
243	232	17.6±0.61	137	174	13.1±0.61	105
273	239	18.3±0.76	129	185	13.0±0.74	101
304	243	19.6±0.87	116	186	14.4±0.71	94
334	247	17.9±0.89	108	189	14.3±0.72	87
365	254	15.8±0.77	94	188	14.4±0.74	86
395	258	15.6±0.76	75	195	15.4±0.86	71
425	263	19.1±1.24	54	192	14.8±0.93	57
455	269	18.0±1.35	39	195	15.2±1.02	49
		18.0±0.85			14.8±0.76	

cent less than those of the males in the earlier generations, as the position of graph B indicates.

A comparison of graph B with graph C in figure 7 shows that the body-weight increase with age in the males of the later generations of the inbred strain was, on the whole, very similar to that in the males of the series of stock Albinos reared in 1913 to 1915 as controls for the inbred strain: stock males grew somewhat



more vigorously during the adolescent period, but they were not as heavy as the inbred males in adult life. Since the inbred males were fully as large as the males in the stock series that had been reared under much more favorable conditions of environment and of nutrition, it is evident that continued inbreeding

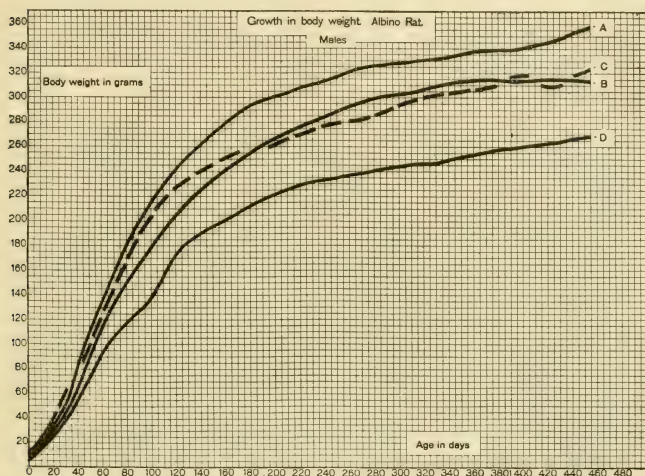


Fig. 7 Graphs showing the increase in the weight of the body with age for males belonging to four series. A, graph for males of the seventh to the fifteenth generations of the inbred strain (series A, B); B, graph for males of the sixteenth to the twenty-fifth generations of the inbred strain (series A, B); C, graph for males of the selected series of stock Albinos reared in 1913 to 1915 as controls for the inbred strain; D, graph for males of the stock series reared simultaneously with the individuals of the twenty-first to the twenty-fifth generations of the inbred strain (data in table 10 and in table 11 of the present paper and in table 13 of 'Studies on inbreeding I,' King, '18).

has not produced a deterioration in the original stock as regards the normal weight increase with age. The males in the seventh to the fifteenth generations of the inbred strain were much superior in body weight to outbred stock males reared under similar environmental and nutritive conditions (compare graph A with graph C in figure 7). Likewise, inbred males of the six-



teenth to the twenty-fifth generations, living for the most part under the handicap of inadequate nutrition, were considerably heavier at all ages than the males in a stock series that were reared simultaneously with them, as a comparison of graph B with graph D in figure 7 shows. The space between these graphs, at the 200-day period, indicates a difference of about 17 per cent in favor of the males of the inbred group.

Growth graphs for various groups of female rats are shown in figure 8.

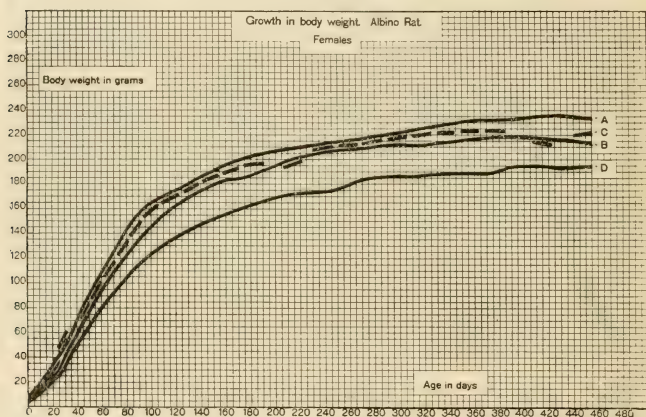


Fig. 8 Graphs showing the increase in the weight of the body with age for females belonging to four series (data and lettering as in table 7).

The growth graphs for various groups of females, shown in figure 8, have the same relative positions as have the graphs for the corresponding groups of males (fig. 7), but they lie somewhat closer together. Inbred females of the seventh to the fifteenth generations, as graph A shows, were heavier at all ages (except thirteen days) than the females of the other groups; in the adult state their average body weights were about 2 per cent greater than those of the inbred females belonging in subsequent generations (graph B). Body weight increase with age

in females of the sixteenth to the twenty-fifth generations of the inbred strain closely followed that of the females in the first series of stock controls (compare graph B with graph C in figure 8). The animals in both of these latter groups were about 14 per cent heavier in adult life than the females in the stock series reared during the past two years (graph D).

In explanation of the remarkably vigorous growth of the animals in the seventh to the ninth generations of the inbred strain it was suggested in the first paper of this series (King, '18) that: "favorable nutritive conditions following a period of semi-starvation greatly increased metabolic activity and so stimulated the growth impulse that the animals attained an unusually large size. After the maximum effect of the stimulus had passed there was a gradual decline to more normal conditions of metabolism and a corresponding decrease in the average size of the individuals." Rats seem to be particularly sensitive to changes in food conditions, more so than is generally supposed, and only by feeding them constantly on a proper diet can their normal weight and fertility be maintained. In light of the valuable researches of McCollum ('18) and his associates, it is evident that the 'scrap' food that the rats received during the period when they exhibited their maximum growth and fertility not only furnished a well-balanced ration as regards the basic food stuffs, but that it also gave a sufficient quantity of the essential accessory foods, 'fat-soluble A' and 'water-soluble B,' to greatly stimulate the growth processes. The experimental diets recently used in our colony have very evidently been deficient in 'fat-soluble A.' As a result the rats have shown marked evidence of malnutrition, although they have received an abundance of food. By rectifying the mistakes of the past and feeding the animals on a properly balanced ration, it is hoped that body growth will again respond to the stimulus of adequate nutrition and that it will be possible to obtain inbred animals that are as large as those in the seventh generation. As after twenty-five generations of brother and sister matings the animals in the inbred strain were fully as large as were the best stock animals obtainable, it is evident that close inbreeding does not inevitably

cause a decrease in body size, as Darwin ('75, '78), Crampe ('83), Ritzema-Bos ('93, '94), and others have asserted. Inadequate nutrition, seemingly, is far more detrimental to body growth than is close inbreeding, even when continued over many generations.

#### VARIABILITY IN THE BODY WEIGHTS OF INBRED RATS

At the end of fifteen generations of brother and sister matings the rats in the inbred strain were over 96 per cent homozygous, according to the calculations of Fish ('14). Animals of the later generations, which had attained a degree of homozygosity probably greater than that ever before reached by any group of laboratory mammals, might be expected, perhaps, to show a very great uniformity in body weight at different age periods, if the body weight increase with age in the rat is entirely dependent on the action of genetic growth factors. But just as the rate and extent of body growth in this animal seems to be largely a matter of environment and of nutrition, so also the variations in body weights at different age periods are apparently greatly influenced by these conditions. As it is impossible, at present, to distinguish the variability due to environmental and nutritive action from that resulting from a difference in the genetic factors for body growth, one can only calculate the total amount of variability in given groups of animals and then, by comparison, determine the relative variability of the groups. No very definite conclusions can be drawn regarding the effects of close inbreeding on the variability in the body weight of the rat until the animals can be kept under environmental and nutritive conditions that are so uniform that their effect is practically constant and therefore negligible.

In order to obtain some idea regarding the relative extent of variability in the body weights of the animals in various generations of the inbred strain, coefficients of variability, with their probable error, were determined for the body weights of the individuals in the sixteenth to the twenty-fifth generations of each of the two inbred series and for the weights of the animals in the two series combined (A, B). These coefficients, with

their probable error, were calculated from the data summarized in tables 8, 9, and 10 according to the formulae given by Davenport ('14); they are shown in table 12.

During early postnatal life, as the coefficients in table 12 show, the females in both inbred series were slightly more variable in body weight than were the males, but after thirty days of age the males, as a rule, were the more variable. Variability

TABLE 12

*Showing the coefficients of variation, with their probable error, for the body weights at different ages of the two series of inbred rats (sixteenth to the twenty-fifth generations, inclusive)*

AGE	SERIES A		SERIES B		COMBINED SERIES (A, B)	
	Males	Females	Males	Females	Males	Females
<i>days</i>						
13	12.6±0.45	13.5±0.56	12.2±0.54	12.2±0.44	12.4±0.36	12.9±0.33
30	12.1±0.43	11.9±0.50	14.3±0.63	14.4±0.52	13.5±0.37	13.3±0.36
60	22.9±0.82	18.0±0.75	16.3±0.71	16.4±0.60	20.6±0.57	17.1±0.46
90	20.1±0.72	15.8±0.69	14.8±0.65	14.0±0.53	18.4±0.51	15.5±0.44
120	19.1±0.68	13.7±0.58	14.3±0.63	11.9±0.44	16.1±0.45	12.7±0.35
151	14.5±0.52	10.2±0.45	13.7±0.60	10.7±0.39	14.2±0.39	10.5±0.30
182	13.3±0.48	9.1±0.39	12.6±0.59	12.3±0.44	13.1±0.36	10.4±0.29
212	13.3±0.49	9.6±0.43	12.0±0.53	8.7±0.33	12.8±0.43	9.2±0.39
243	13.3±0.50	9.8±0.45	11.3±0.51	8.5±0.32	12.4±0.36	9.3±0.27
273	12.6±0.49	9.9±0.47	10.9±0.50	9.1±0.35	11.8±0.35	9.4±0.29
304	11.3±0.45	10.8±0.53	10.2±0.48	8.6±0.34	11.2±0.34	9.7±0.30
334	12.3±0.52	10.1±0.50	11.2±0.55	7.9±0.32	11.7±0.37	8.9±0.28
365	12.3±0.54	10.3±0.53	12.0±0.62	9.0±0.38	12.2±0.45	9.6±0.31
395	12.9±0.61	11.3±0.62	12.4±0.67	9.8±0.44	12.7±0.45	10.5±0.36
425	14.2±0.73	10.2±0.59	12.9±0.77	10.5±0.50	13.8±0.54	10.7±0.39
455	14.3±0.79	11.7±0.73	13.6±0.69	11.5±0.62	14.1±0.59	11.6±0.47
Average.....	14.4±0.58	11.6±0.55	12.8±0.60	10.9±0.43	13.8±0.43	11.3±0.35

was at its maximum for both sexes at the sixty-day period, and then tended to decrease with advancing age for some time. In table 12 the average coefficient for the male group in each of the two inbred series, taking all ages together, exceeds that for the corresponding group of females by over two points. Since this difference is over three times the probable error, it is sufficiently large to indicate that the males had a greater range of vari-



ability in body weight than had the females. Coefficients of variability for the body weights of the individuals in the earlier generations of the inbred strain (King, '18; table 15), and also those for various series of stock Albinos (Jackson, '13; King, '15), all show that the males are more variable than the females. Such a relation between the sexes as regards the variability in their body weights would seem to be a characteristic of the albino strain of rats in general, and from the results obtained in the present study it is evident that this relation has not been changed by twenty-five generations of close inbreeding.

Males in the sixteenth to the twenty-fifth generations of the A series of inbreds had a somewhat greater range of variability in body weight than had the males of the B series, judging from the relative size of the coefficients for the two series as given in table 12. Between the average coefficients for the two series there is a difference of 1.6 points in favor of the males of the A series; a similar relation between the two series existed also at an earlier period (King, '18; table 15). Throughout all generations of the inbred strain, therefore, the range of variability in body weights was greater in the males of the A series than in those of the B series. This difference persisted even during the periods when body growth and variability were greatly influenced by environmental and nutritive conditions.

A comparison between corresponding coefficients for the females of the two inbred series (table 12) shows that, as a rule, the females of the A series were slightly more variable in body weight at different age periods than were the females of the B series, but, taken as a whole, the one group of females was about as variable as the other, since the difference between the average coefficients for the two groups is only 0.7 point. As the study of variability in the females of the earlier generations of the inbred strain led to the conclusion that "the range of variability in body weights was practically the same for the females of the two inbred series," it is evident that long-continued inbreeding has not altered the relative variability of the females in the two inbred series any more than it has that of the males.



Table 12 shows that in each inbred series the coefficients of variability for both sexes decrease in size with advancing age until the animals attained an age of about 300 days, and then tend to become somewhat larger; a similar change in the size of the coefficients at various age periods was also noted for the animals in the earlier generations of the inbred strain as well as for those in the two stock series reared as controls. After reaching the height of their reproductive activity at the age of from seven to ten months, certain individuals, especially males, tend to accumulate an excess of adipose tissue; while other individuals, even members of the same litter, will show little change in body weight for a period of several months, or they may even decline steadily in body weight although they are apparently in good physical condition. The increased variability in the body weights of older rats is, therefore, due in great part to the accumulation of a greater or less amount of adipose tissue; it is not a growth phenomenon comparable to that shown during early postnatal life.

In order to make a closer analysis of the relative variability in the body weights of animals in successive generations of the inbred strain, coefficients of variability were calculated from the body-weight data for the animals in three generations combined as summarized in table 7. This series of coefficients is shown in table 13.

In table 13 the average coefficients for the male groups comprising the individuals of the sixteenth to the twenty-fourth generations vary by less than one point, so it is evident that in the later generations of the inbred strain the variability in the body weights of the males did not decrease with the advance of inbreeding, as was the case in the earlier generations (King, '18; table 16). The series of coefficients for the males of the twenty-fifth generation are, as a rule, smaller than the corresponding coefficients for the males of the preceding generation group. But the difference between the average coefficients for the two groups is less than three times the probable error, so it cannot be considered as significant, especially as the number of body-weight records used in calculating the coefficients for the animals

TABLE 13

*Showing the coefficients of variation, with their probable error, for the body weights at different ages of inbred rats of the two series (A, B) separated into groups according to the generation to which the individuals belonged*

AGE	MALES				FEMALES			
	Generations 16-18	Generations 19-21	Generations 22-24	Generation 25	Generations 16-18	Generations 19-21	Generations 22-24	Generation 25
<i>days</i>								
13	12.5±0.06	13.7±0.72	12.8±0.61	7.7±0.64	13.5±0.72	13.8±0.67	12.8±0.62	7.1±0.58
30	13.3±0.70	13.1±0.69	13.4±0.64	6.9±0.57	14.7±0.79	11.8±0.57	13.6±0.66	7.6±0.62
60	17.9±0.94	17.0±0.90	15.1±0.72	18.8±0.76	15.8±0.80	13.7±0.67	18.3±0.89	14.6±1.20
90	14.2±0.75	15.6±0.82	17.5±0.84	12.1±1.00	11.1±0.64	12.3±0.64	15.7±0.78	11.1±0.94
120	11.8±0.63	13.9±0.73	14.3±0.68	10.5±0.87	11.1±0.59	10.3±0.52	11.6±0.59	8.2±0.67
151	12.4±0.65	11.9±0.63	12.0±0.57	10.2±0.85	10.2±0.54	7.9±0.39	9.0±0.46	8.8±0.77
182	11.4±0.61	11.1±0.59	12.1±0.58	9.6±0.80	9.4±0.51	6.4±0.33	8.6±0.43	6.7±0.56
212	11.1±0.61	10.7±0.58	13.2±0.64	10.1±0.83	8.4±0.47	6.8±0.35	8.6±0.43	7.6±0.66
243	10.4±0.59	10.9±0.60	12.2±0.59	9.9±0.82	8.4±0.49	7.0±0.38	8.0±0.40	7.1±0.62
273	10.3±0.63	11.3±0.64	11.4±0.56	9.9±0.85	9.8±0.59	6.9±0.38	7.1±0.38	5.9±0.52
304	9.9±0.61	10.1±0.59	10.3±0.52	11.0±1.00	9.6±0.60	8.1±0.55	8.1±0.42	7.8±0.71
334	10.4±0.69	11.8±0.71	10.6±0.56	10.9±0.99	9.3±0.59	7.6±0.44	8.1±0.44	6.3±0.58
365	11.4±0.85	12.6±0.81	11.3±0.61	10.6±0.98	9.7±0.66	8.8±0.53	8.6±0.48	7.2±0.66
395	15.9±1.37	12.4±0.85	10.4±0.58	10.8±0.97	10.4±0.76	8.9±0.56	8.8±0.52	11.1±1.00
425	11.2±1.05	15.8±1.22	10.6±0.65	12.2±1.21	9.8±0.80	8.3±0.58	9.6±0.56	9.9±1.05
455	13.3±1.35	14.1±1.12	11.7±0.78	13.0±1.32	11.9±1.25	9.4±0.72	10.7±0.67	8.6±0.99
Average.....	12.3±0.79	12.8±0.76	12.4±0.63	10.9±0.86	10.8±0.67	9.2±0.51	10.4±0.55	8.5±0.76

of a single generation was only about one-third of that used for a group of three generations.

The average coefficients for the three groups of females comprising the animals in the sixteenth to the twenty-fourth generations of the inbred strain are all lower than those for the corresponding groups of males (table 13), and they also fail to show a significant decrease in size as inbreeding advanced. The average coefficient for the body weights of the females in the twenty-fifth generation is considerably smaller than that for any of the three generation groups, but here also no definite conclusion seems warranted, since the small number of records on which the coefficients are based may be responsible in great measure for the result.

The animals in the seventh to the fifteenth generations of the inbred strain lived under environmental and nutritive conditions that were fairly uniform and seemingly very favorable to growth and to fertility. The body weights of these individuals showed a slow decrease in variability with the advance of inbreeding, as the relative size of their coefficients of variability indicates (King, '18; table 16). During early life the rats in the sixteenth and seventeenth generations lived under the same environmental and nutritive conditions as the animals of the preceding generations, and at this time they were all seemingly somewhat less variable in body weight than were the individuals in the fifteenth generation. Before the weight records for these rats were completed, a change in diet became necessary, as 'scrap' food of the required quality and quantity could no longer be obtained. The effects of the change in food became very apparent in the course of a few weeks, and, as individual rats responded differently to the altered conditions of nutrition, there was a marked increase in the variability of the body weights in the animals of all ages. When the coefficients of variability were calculated from the series of body-weight data obtained for the animals in the sixteenth to the eighteenth generations, they were found to be somewhat larger than those for the animals in the fifteenth generation, as was expected from the observed appearance of the animals. The animals in the later generations of the inbred

strain have shown a variability in body weights considerably greater than that found in any group of inbred animals since the tenth generation.

By comparing the corresponding coefficients for the two series of outbred stock Albinos that were reared in the colony on different diets, one can determine whether the variability in the body weights of these animals was influenced by the nutritive conditions under which they lived. By a further comparison of these coefficients with those for the animals in the later generations of the inbred strain, it will be possible to determine whether the increase in the variability of the inbred animals was due to altered conditions of nutrition or to the effects of long-continued inbreeding.

All of the stock Albinos reared in 1913 to 1915 as controls for the inbred series were fed on 'scrap' food. As has already been recorded (King, '15; table 4), the coefficients of variability for the body weights of the fifty males in this series range from 10.2 to 17.0, with an average of 13.6 for the entire group, taking all ages together; coefficients for the fifty females vary from 8.9 to 15.7, with an average of 11.5 for the entire group.

The second series of stock controls was reared in 1916 to 1918 simultaneously with the inbred rats of the twenty-first to the twenty-fifth generations, and they, as the inbred rats, were fed on various experimental diets. These stock Albinos came from the same general stock colony that furnished animals for the first series of controls, so the coefficients for the two series are strictly comparable. An examination of the coefficients for the body weights of the rats in this control series, as given in table 11 of the present paper, shows that all of them are much larger than the corresponding coefficients for the animals of the first stock series, while the difference between the average coefficients for the two series is over four times the probable error. It is evident, therefore, that the rats in the second series of stock controls were much more variable in their body weights at all age periods than were the animals in the first stock series. Since both of these stock series were outbred, the increased variability in the animals of the second series cannot be attributed to the



effects of inbreeding; nor can it be ascribed to a difference in the genetic constitution of the two series of animals, since no new 'blood' was introduced into the general stock colony from 1913 to 1917. From the evidence given, one seems warranted in assuming that the marked difference in the variability of the two series of stock animals was due, in great part, to the effects of changed conditions of nutrition which so greatly influenced the body growth of the individuals in the second series. It is probable also that the extremes of temperature to which many of these rats were subjected also affected their variability in body weight to some extent, although the effects of temperature changes were very much less than those of nutrition.

Since the variability in the body weights of outbred stock Albinos was seemingly greatly affected by nutritive and environmental factors, one would naturally conclude that these factors would likewise influence the variability in the body weights of inbred animals reared simultaneously with and under the same conditions as the stock Albinos. The increased variability in the inbred animals of the sixteenth to the twenty-fifth generations is, on this assumption, the result of environmental and nutritive action, and it cannot be cited in support of Walton's ('15) contention that continued inbreeding tends to increase variability. It is interesting to note in this connection that a comparison between the average coefficients for various groups of inbred rats and those for stock Albinos indicates that changed conditions of nutrition produced a much greater effect on the variability in the body weights of stock Albinos than it did on that of the animals in the later generations of the inbred strain.

In this experiment, owing to the action of environment and of nutrition, it is impossible to determine the changes, if any, that inbreeding per se produced on the variability in the body weights of the animals in the later generations of the inbred strain. This study of variability is of value, therefore, mainly because it shows that in the later generations of inbreds there existed between the two series (A and B), and between the two sexes, the same relative variability in body weights as that found in the earlier generations. Twenty-five generations of brother



and sister matings have not, seemingly, altered the relative variability in the strain, whether the total amount of variability has been influenced by inbreeding cannot be determined until it is possible to rear a number of generations of these animals under uniform conditions of environment and of nutrition.

#### GENERAL CONCLUSIONS

As a whole, this experiment has shown that the closest form of inbreeding possible in mammals, the mating of brother and sister from the same litter, is not necessarily inimical either to body growth, to fertility, or to constitutional vigor, provided that only the best animals from a relatively large number are used for breeding purposes. Selection, seemingly, is able to hold in check any tendency that inbreeding may have to bring out the undesirable, latent traits inherent in the strain.

In the course of this investigation it has been shown that adverse conditions of environment and of nutrition produce far more detrimental effects on growth and fertility in the albino rat than does inbreeding. These factors, apparently, do not alter the genetic constitution of the individual, since the animals soon resume their normal growth and fertility when environmental and nutritive conditions are again favorable.

The sex ratio in the rat is seemingly a character that is amenable to selection, since through this process the inbred strain has been separated into two lines: one line (A) showing a high sex ratio, the other line (B) showing a low sex ratio. The effects of selection on the sex ratio seem to be limited, however, since there has been no cumulative effects of the selection, although the two lines have been kept distinct for eighteen successive generations. Whether it will be possible to change the sex ratio in the two lines by reversing the selection is the chief problem in view in the continuation of this work.

Throughout the entire course of this investigation there has been a great similarity between the two inbred series as regards the variability in the body weights of the animals at different age periods. In the earlier generations the variability in body

weights seemed to decrease with the advance of inbreeding, but in the later generations the variability was greatly influenced by environmental and nutritive conditions. Until these latter factors can be controlled, it will not be possible to draw any definite conclusions regarding the effects of inbreeding per se on the variability in body weights.

#### SUMMARY

1. The data given in the present paper show the growth and variability in the body weights of 296 males and of 310 females belonging in the sixteenth to the twenty-fifth generations of two series (A and B) of albino rats that were inbred, brother and sister from the same litter.

2. Owing to economic conditions, many of these rats were not reared under very favorable conditions of environment and of nutrition, and in consequence they did not grow as rapidly nor did they attain as great a maximum body weight as did the individuals in the earlier generations of this inbred strain.

3. In every generation from the sixteenth to the twenty-fifth the males were heavier than the females at all age periods after thirty days (tables 1 to 4). This result agrees with the finding for the inbred rats of the earlier generations, and also with that for various series of stock Albinos. Apparently, therefore, long-continued inbreeding has not changed the normal body-weight relations of the sexes at any age period for which records have been taken.

4. In the A series of inbreds the rate and extent of growth in body weight were much the same as those in the B series of inbreds: in the adult animals there was a difference of only about 2 per cent in the average body weights of corresponding groups of males and females in the two series (tables 8 and 9; fig. 5 and 6).

5. Close inbreeding for twenty-five generations has not altered the form of the growth graph for the albino rat to any extent.

6. Rats belonging to the later generations of the inbred strain were not as heavy at any age period as were the animals in the earlier generations, but they were much superior in body weight to stock Albinos reared under similar conditions of environment and of nutrition (figs. 7 and 8).

7. Individuals in the sixteenth to the twenty-fifth generations of the inbred strain had about the same average body weight at different age periods as had the individuals of the stock controls reared in 1913 to 1915 under favorable conditions of environment and of nutrition (figs. 7 and 8; compare graph B with graph C). Seemingly, therefore, inbreeding has as yet produced no deterioration in the original Albino stock as regards the rate and extent of growth in body weight.

8. Variability in the body weights of the animals in the later generations of the inbred strain followed the same general trend as that in the animals of the earlier generations and in those of the two stock series studied: in both sexes it increased from birth to sixty days, and then decreased steadily until the animals were about 300 days of age, tending to rise again in older rats (table 12).

9. In the later generations of the inbred strain the males were more variable in body weight than the females. This result agrees with the finding for the animals of the earlier generations and for various series of stock Albinos.

10. In the inbred animals of the sixteenth to the twenty-fifth generations variability in body weights was relatively high, and it did not tend to decrease with the advance of inbreeding as in the earlier generation (table 13).

11. Outbred stock Albinos, reared simultaneously with and under the same environmental and nutritive conditions as the inbred rats of the twenty-first to the twenty-fifth generations, showed a variability in their body weights at all ages much greater than that in the animals of the earlier stock series reared under more favorable conditions of nutrition. It appears, therefore, that the increased variability in the body weights of the animals in the later generations of the inbred strain was due to the action of environment and of nutrition, not to the effect of continued inbreeding.

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Resumen por el autor, Harley Nathan Gould.  
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Estudios sobre el sexo en el molusco hermafrodita *Crepidula*  
plana.

III. Transmisión del estímulo productor de machos por el  
agua de mar.

El molusco gasterópodo *Crepidula plana* pasa durante su vida por una fase de macho, una fase de transición y una fase de hembra. La fase de macho es inestable y se presenta solamente como resultado de un estímulo suministrado por un individuo de la misma especie más grande que el individuo estimulado. El aislamiento completo de los individuos pequeños no desarrollados sexualmente, durante largos periodos, demuestra que bajo tales condiciones no tiene lugar más desarrollo de los caracteres machos que la formación de unas pocas espermatogonias. En su debido tiempo aparecen los caracteres de la hembra. Los individuos pequeños y no desarrollados sexualmente confinados a distancias fijas de 4 a 7 mm. de hembras grandes, impidiéndose de este modo todo contacto, desarrollan en la mayor parte de los casos caracteres del macho en varios estados de madurez sexual. Bajo tales condiciones se producen menos machos y peor desarrollados que cuando los animales pequeños están más cerca del origen del estímulo. Los individuos grandes de *Crepidula fornicata*, una especie próxima a *Crepidula plana*, no inducen desarrollo alguno sobre los individuos pequeños de esta última especie, excepto en unos cuantos casos dudosos. El estímulo que provoca el desarrollo de machos actúa de tal modo que indica que es una substancia que sale de los cuerpos de los individuos grandes de *Crepidula plana*, la cual substancia es difusible en el agua de mar, pero es muy inestable.

## STUDIES ON SEX IN THE HERMAPHRODITE MOLLUSC CREPIDULA PLANA

### III. TRANSFERENCE OF THE MALE-PRODUCING STIMULUS THROUGH SEA-WATER

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ONE TEXT FIGURE

The second paper of this series<sup>1</sup> described a number of experiments showing the instability of the male phase in the marine gastropod *Crepidula plana*. In common with other members of the family Calyptraeidae, *C. plana* passes through a sperm-producing phase during the early part of its life while it is small (up to about 15 mm. in length) followed by a transitional phase (15 to 20 mm.) and later by an egg-producing phase (20 to 40 mm.). Growth goes on with varying degrees of rapidity during life. The functional females are the largest and oldest. The species has a peculiarity in that the development and maintenance of the male phase requires a stimulus from the outside, which is furnished by the presence of a larger individual, usually transitional or female, in the immediate vicinity of the potential male.

The animals are most commonly found in colonies adhering to the inner surface of shells inhabited by hermit crabs. The younger, smaller *Crepidulas* have various degrees of male development, those directly attached upon the shells of the large females as a substratum, or close beside them, being nearly all fully developed males, while those at a distance of 5 mm. or over are more likely to have only partially developed male organs; the degree of development being less in the specimens farther from the source of the stimulus, i.e., the large individuals of the colony.

<sup>1</sup> Gould, 1917, II.

In a group in which there are no females and all the members are less than 10 or 12 mm. in length, there are seldom any adult males; the majority being, instead, sexually undeveloped (neuter); but often the smaller members of such a group have a rudimentary male development, evidenced by the presence of many spermatogonia in the sex gland, even some spermatogenesis and a rudimentary penis. In fact, wherever two members of the species are attached close together, however insignificant the difference in size between them, the smaller tends to begin male development.

#### ISOLATION OF NEUTERS

The adult male stage is never developed in isolated animals, nor can it be maintained after removal of a male from the colony. Wishing, however, to determine whether any partial development of male characters would take place in completely isolated specimens, the writer allowed young neuter animals to attach themselves to the inner surface of glass vials, one to each vial. These were all kept in salt-water aquaria. Selection of the specimens for the experiment was made with care from hermit shells containing only a few small *C. plana*. Each was examined with a lens, and only those quite devoid of rudimentary male characters were used. After isolation a few specimens were taken from time to time, examined, then fixed and sectioned for study of the gonad. At the beginning all were from 5 to 12 mm. in length, and were thus at the size when male development can easily be induced. They grew during the period of isolation, and the last lot, taken at fifty days, were much larger. Slides were made from twenty-four specimens; two at twenty-two days' isolation, four at twenty-four days, three at twenty-six days, five at thirty-three days, five at forty-three days, and five at fifty days. The results may be summarized as follows:

External male characters: In three animals only, two twenty-four days and one at thirty-three days, there was a very small stump at the spot where the penis forms. No other external signs of the male condition appeared.



Gonad: In three cases there were a few spermatogonia in the sex gland; one at twenty-two days, one at twenty-four days, and one at forty-three days. None of these corresponded with any one of the three having a rudimentary penis. In sixteen cases the gonad was inactive (containing only primordial male and female cells). The remaining five were the animals sectioned after fifty days' isolation. They had passed from the neuter to the incipient female condition, having various stages in early growth periods of oocytes, and had grown considerably in size, being now from 14 to 23 mm. in length.

A similar record was made of males removed from colonies and kept isolated in vials. As was shown in a former paper, all males lose their male characters after removal from the colonies. Four samples were taken from the vials after thirty-six days, four after forty-six days, four after fifty-three days, and four after sixty days. There was no resumption of spermatogenesis or redevelopment of external male organs after the degeneration in any case. The only hint of any such activity was the presence of a few dividing spermatogonia in the gonad of one isolated forty-six days. It should be recalled that previous experiments demonstrated the ability of degenerate males to reassume the functional male state under stimulus from larger individuals.

It is thus indicated that the gonads of isolated small specimens may produce a few spermatogonia, but proceed no further toward spermatogenesis; and the spermatogonia so formed later degenerate, as sections show. The isolation experiment is meant to clear the way for others, i.e., to show, in cases where partial male development is induced under weak stimulus, how much of this is due to internal causes. The writer concludes that rapid spermatogonial multiplication, formation of spermatocytes, or any later stage of spermatogenesis is an indication of an external stimulus.

In previous experiments where they developed male characters under observation, the neuters were placed as closely as possible to the larger animals. Only in this way could the stimulus be clearly shown. The writer failed to find positive evidence

of a stimulating secretion thrown into the sea-water. The question arose whether physical contact is necessary for the transference of the stimulus.

#### STIMULUS WITHOUT CONTACT

A simple apparatus (fig. 1) was devised to hold a large female *Crepidula* at a definite distance from a small neuter without allowing them to touch or to move farther apart. The female was removed from the inner surface of a hermit crab's shell and allowed to attach herself to the concave surface of a watch crystal. The small neuter was placed on the floor of the flat-bottomed depression in a hollow-ground slide. Mosquito netting was fastened over the depression to prevent the neuter from escaping.

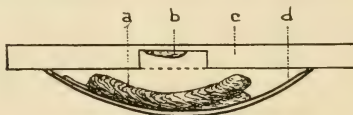


Fig. 1 Diagram showing method of preventing contact between specimens. *a*, large female; *b*, small neuter; *c*, depression slide; *d*, watch crystal.

The depression slide was inverted and fastened over the watch crystal containing the large female; leaving the neuter animal, imprisoned in its cell, at a distance of from 4 to 7 mm. from the top of the female's shell. There was no possibility of contact, yet there was little hindrance to diffusion currents in the sea-water between the two. The variation in the distance between female and neuter was due to irregularities in the curvature of the watch crystal and in depth of depression of the slide. The average distance was 6 mm.

After various periods, samples of the originally neuter *C. plana* were fixed and sectioned. The results are tabulated below (table 1). Those specimens the gonads of which showed any male development beyond the mere presence of spermatogonia are marked 'male.' 'Inactives' are specimens with primordial germ cells only, or with these plus spermatogonia. 'Females' are animals where some development of oocytes could be detected.

TABLE 1

DURATION	NUMBER OF SPECIMENS	NUMBER OF MALES	NUMBER OF INACTIVES	NUMBER OF FEMALES
<i>days</i>				
14	7	4	3	0
15	20	11	3	6
17	21	14	3	4
21	19	14	5	0
Total.....	67	43	14	10

Thus, forty-three out of sixty-seven, or about 64 per cent, showed spermatogenetic activity of some sort more than isolated neuters show. Classifying these forty-three with regard to degree of male development, we have: fully developed testis, twenty-five; testis containing sperm, but with some missing stages of spermatogenesis, four; testis developed as far as spermatids, eight; spermatogonia and spermatocytes, two; spermatogonia in multiplication period, four.

The occurrence of incipient female development in some of the specimens will be understood if we assort them all in the order of their size, indicated by the length of the shell in millimeters. This is done in table 2.

All those having early stages of developing oocytes ('female') are seen to be among the larger animals used for the experiment.

TABLE 2

LENGTH	NUMBER OF MALES	NUMBER OF INACTIVES	NUMBER OF FEMALES
<i>mm.</i>			
8	6	0	0
9	10	2	0
10	12	2	0
11	6	1	0
12	5	3	1
13	3	1	2
14	1	3	1
15	0	2	3
17	0	0	1
18	0	0	1
20	0	0	1

Female development is much slower than male, and it is likely that the most of these animals were already in the course of female differentiation when selected as neuters. The percentage of 'inactives' is also greater among the larger specimens. It has been evident to the writer from many observations that the tendency to male development under stimulus gradually wanes as the period approaches when female development may set in. It is, however, sometimes possible to superimpose male on early female development, as shown in the former paper.

In the watch-crystal experiment forty-three animals out of a possible sixty-seven showed some degree of male activity in the sex gland, twenty-five of them being fully developed males. Compare this with the result obtained when neuters were placed on and closely around females. In the latter case (from records in previous paper) fifty-one out of a possible fifty-three showed some degree of male development, and thirty-four of them were adult males. It is clear that more males develop when the neuters are close to the source of the stimulus than when separated by several millimeters; and furthermore, the difference in the results of these two experiments cannot be adequately set forth in tabular form. Examination of the gonad under the microscope shows it more strikingly. Many marked 'adult testis' in specimens from the watch-crystal experiment are only a fraction of the size of the gonads developed in those placed close to or on the large females. There are often signs of arrested development in the former, shown by the paucity or absence of some stages of spermatogenesis.

An examination of the small individuals in a large number of normal colonies shows about 62 per cent adult males (determined from external characters). By placing neuters on and close to females, about the same percentage of adult males was obtained, and this could have been raised considerably by rejecting all those specimens which had moved several millimeters from the females during the course of the experiment. In the watch-crystal experiment only about 38 per cent became adult males.

The development of the male phase by neuters imprisoned in depression slides thus shows that the male-producing stimulus is

able to act in the absence of physical contact and through several millimeters distance in sea-water. A comparison with other experiments indicates that fewer and less fully developed males are produced under such conditions than when the stimulus acts more directly.

The writer has tried several times to determine whether a large female of *Crepidula fornicata*, another species of the same genus, could furnish the stimulus for male development in a small neuter *Crepidula plana*. The experiment has been difficult to carry out, as the little *C. plana* neuters were generally crushed by the twisting and turning movements of the great *C. fornicata* before sufficient time elapsed to make the experiment valuable. The writer has, however, slides made from thirty-two *C. plana* selected as neuters and kept near the *C. fornicata* for various periods. Of these, twenty-one remained entirely neuter and six became incipient females. The remaining five show traces of male development. Two of these must be counted out because the microscopic appearance of the gonad shows that the few products of spermatogenesis there must have been formed and further activity must have ceased before the experiment began. This leaves only three which seem to have developed any male characters during association with *C. fornicata*, and they are as follows:

a. Penis partly developed and small testis as far as spermatids, not very active. Time, seventeen days.

b. No penis. A few spermatogonia and spermatocytes. Time, eleven days.

c. No penis. Spermatogonia and a few spermatocytes. Time, eight days.

Thus, there are no adult males developed out of twenty-six neuter specimens (leaving out of consideration those which had begun female differentiation), but there are three with partial male development during the experiment. This result is rather perplexing. One would naturally expect either an appreciable proportion of males, if the *C. fornicata* exerted any influence, or none at all, if they did not. However, we may draw the conclusion that the male-producing stimulus is not due to any general



change in the medium (sea-water) caused by *C. plana* which would be similarly caused by other species. The *C. fornicata* females used for the experiment were larger than the largest *C. plana* females, and would be expected to throw into the sea-water at least as much of the general katabolic products, for instance, as the latter, yet they had almost no effect in stimulating development of the testis.

It should be emphasized that the power of large animals of the species *C. plana* to stimulate spermatogenesis in the smaller is not limited to females. A number of unusually large males were removed from a colony and imprisoned in a watch crystal with nine small neuters. In eighteen days five of the nine showed some degree of male development, mostly immature. The large males were in the meantime losing their male characters. They were kept forty days after this losing all signs of maleness and growing larger. A second lot of nine small neuters was placed with them. In sixteen days eight of the nine had some degree of male development, averaging nearer the mature male phase than the first nine. The numbers are too small to speak for the relative effectiveness of large males and large transitionals, but show the ability of both to produce the stimulus.

#### SUMMARY

The stimulus passing from larger to smaller *Crepidula plana*, causing the latter to assume and retain the male phase, can be transmitted for several millimeters through sea-water, though its effectiveness is reduced at this distance. Indication that the stimulus may be given faintly by *Crepidula fornicata*, a related species, was given in only three out of twenty-six cases.

The stimulus acts in such a manner as to suggest that it is a specific substance given off from the bodies of the animals, diffusible in sea-water, but very unstable.

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## UROLEPTUS MOBILIS ENGELM.

### II. RENEWAL OF VITALITY THROUGH CONJUGATION

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ONE CHART AND ONE FIGURE

In a previous paper I have described the morphology and the cytology of division and conjugation stages of the rare hypotrichous ciliate which forms the subject of the present paper.<sup>1</sup> Lending itself admirably to the cultural method which has been employed, *Uroleptus mobilis* is the most satisfactory organism for experimental work I have yet encountered. Paedogamous conjugation, in epidemic form, occurs readily, under the proper conditions, in the culture medium. Ex-conjugants, upon isolation, live and thrive in this culture medium in practically 100 per cent of cases—a rare phenomenon among the hypotrichs. Asexual reorganization, or parthenogenesis, called by Woodruff and Erdmann 'endomixis', occurs at fairly definite periods, under the protection of a cyst membrane. Such reorganizations, therefore, are advertised by the form assumed by the organisms and do not interfere with the study of comparative vitality throughout the life cycle.

Starting with a single individual which was isolated immediately after conjugation, on November 16, 1917, I have followed the life history of thirteen different series, all beginning as ex-conjugants of pairs of normally conjugating individuals, and all were progeny of the original ex-conjugant which was isolated November 16, 1917. Three additional series, derived from encysted individuals, have also been studied in similar isolation cultures. The different series were started at various periods

<sup>1</sup> Calkins, 1919. *Uroleptus mobilis*, Engelm. I. History of the nuclei during Division and Conjugation. Jour. Exp. Zool., vol. 27, no. 3, p. 293.

of the life history of parental series and at different stages of vitality, so that I have abundant data for the study of comparative vitality of parent and offspring. It is with pleasure, based upon admiration for the genius of that gifted pioneer in this field of research, Edouard Maupas, that I can say these data convincingly prove the truth of his conclusions that conjugation in ciliates restores vitality and prevents the phenomena accompanying 'old age' which we include under the terms senescence and natural death.

#### METHODS AND RECORDS

On October 3, 1917, a rich culture in an old hay infusion was found to contain a large number of individuals of *Uroleptus mobilis*. The normal structure of this rare ciliate is described and its systematic position given in my earlier paper. It is illustrated again in figure 1 of the present paper. Attempts were immediately made to cultivate the organism on the usual hay-infusion culture medium. A better medium was obtained by mixing boiled flour water, hay infusion, and spring water, and this was used until the middle of January, 1918. It was in this medium that the first pairs of conjugating individuals were found and isolated, giving the ex-conjugant destined to form the first, or A series. A still better medium was substituted on January 18th. This was obtained by boiling 100 mg. of chopped hay with 130 mg. of flour in 100 cc. of spring water, and diluting this, when twenty-four hours old, with an equal part of fresh spring water. This standardized medium, made fresh each day, has been used exclusively throughout the experiments.

#### *Series and lines*

As used here, the term 'series' is applied to an aggregate of individuals, all derived from a single individual and representing its protoplasm. An ideal way to study vitality of such protoplasm at different age periods would be to follow all of the progeny throughout the life cycle. As this is obviously impossible, I follow as many individual representatives of that protoplasm as time and space will permit. My practice is as follows: When

an initial individual divides, each of the two cells is isolated as the beginning of a 'line;' when these divide, two more lines are started, and one more is added at the next division. Each day a record is made of the number of divisions during the twenty-four hours in each line, and a single individual is isolated with a capillary pipette from each line and transferred to fresh culture medium. The vitality is measured by the division rate, the average number of divisions per day in all five lines for a given period representing the vitality of the series for that period. I have daily records, extending from November 16, 1917, to date, of sixteen series and eighty lines of *Uroleptus*.

### *Conjugation tests*

In every line of a series at the time of the daily isolation there are two or more individuals in the culture dish according to the number of divisions that have occurred. After one is transferred the unused individuals are either thrown away or placed in a similar culture dish containing about 1 cc. of the fresh culture medium. Representatives of all five lines of a series are collected in this way and stored in a moist chamber as 'stock.' Here they accumulate by division until a large number are present. Once a week this stock is washed into a Syracuse dish containing several cubic centimeters of fresh culture medium and set aside as a conjugation test. The number of individuals increases rapidly until, in three or four days, there are many hundreds or even thousands of organisms. These Syracuse dishes are examined carefully every other day for a period of two weeks and without any fresh medium being added. During these two weeks the limited food is gradually exhausted, and by the end of the period conjugation would have occurred if the internal conditions of the organisms were suitable for it. The date of the appearance of conjugations is recorded and the extent of conjugations, up to epidemic frequency. At the end of the period the individuals are small, inactive, and starved, and, other tests being under way, they are discarded.

*Encystment tests*

Encystment, with its accompanying asexual reorganization, has never occurred in my isolation culture dishes. It does occur, however, at certain periods of the life cycle in the Syracuse dishes during the conjugation tests. Once encysted, the organism cannot be coaxed out until after a longer or shorter period in a dried state. Such encystments are particularly abundant just prior to the first epidemic of conjugation in a series, and, in some tests, practically all of the individuals encyst without conjugation. In such cases the Syracuse dishes are set aside and the culture medium is allowed to evaporate. Such Syracuse dishes with the dried cysts are then stacked away for future experiments. After several weeks or months of storage, fresh culture medium is added to the Syracuse dish containing the dried cysts. In some cases the reorganized individuals emerge by the end of a week; in others, two or three weeks may be required. Series B and M were derived from such encysted individuals. The cytology of reorganization during encystment will form the subject of a later paper.

*The measure of vitality*

Reproduction by division is an indication that cell structures are functioning normally, and the rate of division is an index of the condition of metabolic activities of the protoplasm, or a numerical index of the protoplasmic vitality of a series for a given period. The relative activity of a given protoplasm at different periods or of parent and offspring protoplasm for the same period may be obtained by averaging the number of divisions per day in a series for successive periods of the same length of time, e.g., five days, ten days, or sixty days. If we compute the average division rates in this way for successive ten-day periods from start to finish of a series and plot the results, we obtain a curve indicating the relative vitality of the protoplasm of a series at different periods throughout the life cycle. Furthermore, since all series and all lines are fed daily at the same time and with the same standardized culture medium, if we plot a number of curves representing different series which have originated at different



times for identical calendar periods, we can tell whether fluctuations in vitality are due to conditions of the environment or to inherent vitality of the protoplasm. If due to environmental conditions, a correction may be made which will indicate, approximately, the comparative vitality, had the environmental conditions been normal.

Again, if we plot the curves for different series and start them all from the same ordinate, we have a means of measuring the vitality of different series at similar stages of the life cycle. Such curves enable us to find the cyclical incidence of important phases of the life history, such as conjugation and encystment, and to establish the limits within which they occur.

These methods have been used in working out the results described in the following pages. In maintaining the cultures and keeping up the records, as well as in working up the statistical data, I have been fortunate in having the assistance from time to time of Miss Mabel L. Hedge and of my colleague Prof. Louise H. Gregory, whose helpful interest I gratefully acknowledge.

#### GENERAL HISTORY OF THE CULTURES

The fact should be emphasized at the outset that these experiments deal, in the main, with one bit of protoplasm which emerged from the processes of conjugation on November 16, 1917, reproduced abundantly by division, underwent paedogamous conjugation repeatedly, underwent encystment, and is still living with a vigor equal to that at the beginning. Some of this protoplasm has been maintained in isolation cultures whereby conjugation has been prevented and in which encystment does not occur. Such protoplasm invariably dies, the phenomenon of natural death being the last stage of a decreasing vitality—a decrease which begins to show early in the life cycle. Other parts of this protoplasm have been allowed to conjugate among themselves, the subsequent isolation cultures showing the effects of such conjugation upon such protoplasm. Still other parts of this protoplasm have been allowed to encyst and to undergo processes of asexual reorganization within such cysts, and the effects of such reorganization have been ascertained.

Also, the fact should be emphasized again, that throughout the entire history of the protoplasm, with the exception of a few weeks at the outset, the same standardized culture medium, made fresh each day, has been used. Waning vitality cannot be attributed to deleterious food conditions, for, on the same day with the same food, one portion of the protoplasm may be at the lowest ebb of vitality, while other portions are in the full swing of metabolic vigor, all portions being equally old in point of time. The difference in vigor between them cannot be due, therefore, to environmental or external conditions, but must be attributed to the internal conditions following conjugation.

This protoplasm has been studied in fourteen different series as follows: The A series, or parent race, started as an ex-conjugant from a pair of 'wild' *Uroleptus mobilis* on November 16, 1917, and died on September 18, 1918, in the 313th generation. The C series, or first filial series ( $F_1$ ), started on February 4, 1918, as an ex-conjugant from a pair of individuals in approximately the 78th generation of the A series. It died out on December 30, 1918, in the 348th generation. The D series, or second filial series, started as an ex-conjugant on March 9, 1918, from the A series in the 137th generation and died out on October 13th in the 271st generation. An E series was also started at the same time from the same source, but was discarded after the 50th generation. The F series, or first  $F_2$  series, started as an ex-conjugant on March 25, 1918, from the C series in the 86th generation (the grandparent A series was in the 155th generation). This series died December 21, 1918, in the 317th generation. The H series, or third  $F_1$  series, started as an ex-conjugant on April 24, 1918, from the A series in the 237th generation, and died out on January 16, 1919, in the 277th generation. The I series, or first  $F_3$  series, started as an ex-conjugant on July 7, 1918, from the F series in the 143rd generation (the  $F_1$  grandparent C series was in the 224th generation and the great-grandparent A series was in the 278th generation). This series is still living in the 321st generation, but has stopped dividing and will die shortly. The J series, or fourth  $F_1$  series, started as an ex-conjugant on August 20, 1918, from the A series in the 311th

generation, and is still living in the 254th generation. The L series, or first  $F_4$  series, started as an ex-conjugant on November 3, 1918, from the I series in the 199th generation (the grandparent F series was in the 294th generation, the great-grandparent C series was in the 347th generation, and the great-great-grandparent A series was dead). This series, also, is active, and in the 196th generation. The M series started from a cyst of the F series which had encysted on April 27th while in the 45th generation, remained dry for more than six months, and was recovered from the cyst on November 18, 1918. The N series was started on December 12, 1918, as an ex-conjugant from the J series in the 188th generation. The O series was started as an ex-conjugant on January 10, 1919, from the M series in the 105th generation. The P series was started on January 12, 1919, as an ex-conjugant of the L series in its 115th generation. The Q series was started on January 19, 1919, as an ex-conjugant of the I series in its 316th generation. Finally, the R series was started as an ex-conjugant on January 19, 1919, from the J series in its 245th generation.

In addition to these fourteen series representing one protoplasm, two other series, representing a different initial protoplasm, have been followed throughout the life history. Of these the B series started from an encysted 'wild' *Uroleptus mobilis*. This individual encysted on November 9, 1917, remained dry from December 1st until January 24th, when it was recovered from the cyst. A filial series, the G series, started as an ex-conjugant from the B series in the 115th generation on March 23rd, and died out on January 5th, 1919, in the 291st generation.

In computing the division rate of a series for a given period, e.g., ten days, the number of divisions in all five lines for the ten days are added and the sum divided by five. The value of such an average depends somewhat on the extent of variation in number of divisions in each of the five lines. In table 1 the individual line records for six consecutive ten-day periods from June 27th to August 25th and for all the series under observation during this sixty-day period are given. Since the different series were started from ex-conjugants at different times, this sixty-

TABLE 1

*Actual numbers of divisions in ten-day periods in all lines of series A, C, D, F, H, and I*

	LINES	PERIOD						TOTALS 60 DAYS	AVERAGE DIVISION RATE PER LINE IN 10 DAYS
		6/21-7/6	7/7-7/16	7/17-7/26	7/27-8/5	8/6-8/15	8/16-8/25		
A series.....	1	6	5	10	3	2	0	26	5.86
	2	9	10	12	4	2	1	38	
	3	11	8	12	5	2	0	38	
	4	9	9	10	7	4	0	39	
	5	10	6	13	5	1	0	35	
C series.....	1	11	10	16	5	7	10	59	10.90
	2	11	12	19	6	10	10	68	
	3	12	13	17	10	9	9	70	
	4	8	12	18	11	10	9	68	
	5	8	10	18	7	9	10	62	
D series.....	1	13	13	19	14	6	5	70	12.83
	2	13	15	17	14	11	9	79	
	3	14	15	17	6	10	12	74	
	4	14	15	22	12	13	13	89	
	5	13	15	17	10	10	8	73	
F series.....	1	12	13	20	7	10	12	74	13.16
	2	12	14	21	12	9	12	80	
	3	14	15	20	11	12	11	83	
	4	13	14	19	10	7	13	76	
	5	13	15	19	15	8	12	82	
H series.....	1	16	15	23	12	11	14	91	15.26
	2	15	17	19	14	15	15	95	
	3	14	16	24	12	13	16	95	
	4	13	15	22	12	11	16	89	
	5	13	17	21	11	9	17	88	
I series.....	1	18	20	16	10	12	12	88	17.16
	2	16	23	19	11	14	16	99	
	3	18	23	20	12	15	17	105	
	4	20	24	22	13	17	17	113	
	5	20	23	20	13	17	17	110	

day period covers almost every stage of a typical life cycle. It includes a late stage of the A series and the initial stage of the I series, while the others are intermediate.

The five different lines of a series thus give consistent records in number of divisions in the same calendar period, so that an average of all five lines gives a fairly accurate idea of the vitality of the protoplasm of a series for that period. Table 2 is a list of such averages for all series in the same consecutive calendar ten-day periods.

The averages given in table 2 are based on the actual records of daily divisions in all series, the individuals being isolated daily and fed on the same fresh, standardized culture medium. In some periods, notably in periods 20 and 21, and again in 25, the averages are conspicuously out of proportion with those before and after. These low averages, occurring in all series at exactly the same time, but in different phases of the life cycles, are obviously due to external conditions. The 20th and 21st periods fell on May 28th to June 17th. On the 29th of May the cultures were transferred from New York to Woods Hole, where a different natural water was used in making the culture medium, and the temperature also was considerably lower than it had been in New York. This unusual variation in the sequence of averages makes no difference in the life cycle of a given series, but if we wish to compare similar stages in the cycles of all series, some of which include this period of adverse conditions while others do not, it is obvious that a correction of the lower averages is imperative. Such corrections are made for all series in the 20th and 21st periods by averaging the 18th and the 22nd to obtain the 20th, and the 19th and 23rd to get the 21st. In making such corrections the records of the individual lines are used and the rate for each line is corrected; these corrected line averages are then averaged to obtain the corrected average for the series. The same method is employed for other periods in which corrections are necessary. In table 2 such corrected averages are included in brackets, and the corrected averages are used in plotting curves of the life cycles and for comparison of different series in similar phases of the cycle.



TABLE 2

*Average division rates, all series, in the same ten-day periods*

10-DAY PERIODS	A SERIES	B SERIES FROM WILD CYST	C SERIES FROM A 78	D SERIES FROM A 137	F SERIES FROM C 86	G SERIES FROM B 115	H SERIES FROM A 237	I SERIES FROM F 143	J SERIES FROM A 311	L SERIES FROM I 199	M SERIES FROM CYST OF F 45	N SERIES FROM J 188	O SERIES FROM M 108	P SERIES FROM L 116	Q SERIES FROM I 316	R SERIES FROM J 245
1	9.8															
2	6.6															
3	4.8															
4	5.4															
5	7.8															
6	21.0															
7	18.6	22.4														
8	20.8	21.0	18.6													
9	18.0	17.0	18.8													
10	12.6	14.2	16.2													
11	13.2	14.2	16.8	16.0												
12	14.8	15.6	17.2	18.2												
13	14.6	13.4	15.6	16.0	13.4											
14	13.2	13.0	14.2	17.0	13.8											
15	15.0	14.4	16.4	17.6	16.4	17.4										
16	13.6	13.8	15.6	18.4	19.8	19.4										
17	17.0	13.8	17.4	19.0	20.2	21.0										
18	12.8	15.0	18.0	20.0	19.6	19.6	19.6									
19	7.4	12.2	13.4	15.2	15.8	15.6	16.8									
20	4.0	4.0	5.4	7.6	6.2	7.0	7.6									
	(11.0)		(14.4	16.6	16.8	15.8	17.8)									
21	5.6	5.6	6.2	7.2	9.2	8.0	10.0									
	7.0		(12.8	14.4	14.0	13.6	15.8)									
22	9.0	7.6	10.4	13.4	12.8	12.0	14.8									
23	7.6	4.8	11.4	14.6	14.2	12.0	16.0	18.4								
24	11.2	7.2	17.6	18.4	19.8	18.8	21.4	22.6								
25	4.8	2.6	7.8	11.2	11.0	7.4	10.0	12.4								
			(10.6	12.6	13.8	11.8	16.4	19.4)								
26	2.2	1.0	9.0	10.4	15.2	15.0	18.0	11.8								
27	0.2	1.8	9.8	9.4	12.0	12.6	13.6	15.0	17.2							
28	0.6	0.8	8.6	3.4	11.8	12.2	15.6	15.8	16.6							
29	0.2	0.0	7.6	1.4	10.8	12.4	15.4	14.8	18.6							
30	0.0	0.0	6.0	0.8	12.0	15.2	17.8	17.6	17.4							
31		0.0	6.8	0.4	14.6	16.8	17.8	18.0	20.4							
32	.....	0.0	4.8	0.2	9.8	11.6	11.0	11.6	17.2							
33	.....		2.4	0.0	9.6	13.2	15.4	16.4	15.8							
34	.....		0.6	0.0	12.0	9.2	12.6	17.4	17.2							
35	.....		0.0		8.0	3.6	7.4	14.0	14.6	16.0						
36	.....		0.4		5.8	2.4	2.4	12.8	12.4	13.2	8.4					

TABLE 2—*Concluded*

10-DAY PERIOD	A SERIES	B SERIES FROM WILD CYST	C SERIES FROM A 78	D SERIES FROM A 137	F SERIES FROM C 80	G SERIES FROM B 115	H SERIES FROM A 237	I SERIES FROM F 145	J SERIES FROM A 311	L SERIES FROM I 199	M SERIES FROM CYST OF F 45	N SERIES FROM J 188	O SERIES FROM M 108	P SERIES FROM L 116	Q SERIES FROM I 316	R SERIES FROM J 245
37	.....	.....	0.4	.....	3.8	0.2	1.6	13.8	14.4	16.2	17.0					
38	.....	.....	0.0	.....	2.8	0.2	1.6	20.8	20.6	23.2	24.8					
39	.....	.....	.....	.....	0.0	0.8	0.4	15.8	17.0	18.6	19.8	17.2				
40	.....	.....	.....	.....	.....	.....	1.2	13.0	12.6	16.0	18.0	17.8				
41	.....	.....	.....	.....	.....	.....	0.0	14.2	13.0	16.4	24.6	23.6				
42	.....	.....	.....	.....	.....	.....	.....	9.6	6.6	17.4	18.6	17.8	15.2	16.0		
43	.....	.....	.....	.....	.....	.....	.....	3.0	3.6	18.6	18.8	20.4	22.6	19.8	13.2	19.6
44	.....	.....	.....	.....	.....	.....	.....	0.0	0.8	11.2	13.4	13.8	16.8	14.8	5.8	13.8
45	.....	.....	.....	.....	.....	.....	.....	0.2	0.2	12.6	12.4	13.6	16.6	15.0	1.2	14.0

*Incidence of conjugation and encystment*

Conjugation tests in all series have been carried out at weekly intervals beginning with the sixth ten-day period, during which the standardized culture medium was introduced. The records of these tests give the dates on which pairing occurred. With only one exception, every series derived from an ex-conjugant failed to give any evidence of conjugation before the fiftieth day. The one exception was the I series in which a single pair of conjugating individuals were observed in a test made during the third ten-day period. In the C and D series the first pairing occurred in the fifth ten-day period; in the H and L series the first pairing occurred in the sixth period, and in the J and F series the first conjugation occurred in the seventh and eighth periods. In all of these cases, except the I series, conjugations were of epidemic frequency, i.e., hundreds of pairs were present in the testing dishes. It is apparent, therefore, that the first sixty days, approximately, represent a period of immaturity so far as conjugation is concerned, although, during this period, vitality as indicated by the division rate is at its maximum. After conjugation begins in a series, the succeeding tests are usually positive unless some adverse condition renders the test inconclusive.

During the period of immaturity the history of the conjugation tests was the same for all series. Two or three thousand indi-

viduals would accumulate by division in the Syracuse dishes during the first seven to eight days when food was plentiful. With the transition from rich feeding to starvation, conjugation should occur, provided the protoplasm is ready for it. During this period of immaturity, however, no conjugations take place, and at the end of two weeks the individuals are inactive and greatly reduced in size through starvation.

Conjugation tests made during the period of maturity give a very different result. The individuals multiply as before during the first week. Then they begin to collect in a single group until a dense, white agglomeration is formed which sometimes measures half an inch in diameter. Such agglomerations are invariably followed by an epidemic of conjugations. Since each conjugation test is started with a few individuals of the same series that are left over after a daily isolation, the conjugations that occur must be between closely related individuals of the same age and with an identical previous history.

In diagram 1 the ten-day averages given in table 2 are plotted for the purpose of comparing the life cycles of the first eight series. The ordinates represent the average numbers of divisions of a single line in ten days. The abscissas represent the successive ten-day periods. The positions of the curves in the first period indicate the average division rate of each line during the first ten days of life after conjugation, while the positions of the curves in the tenth represent the average numbers of divisions between the 90th and the 100th days after conjugation, etc. The period during which conjugations occurred to furnish the filial series is shown on the curve of the parental series by a letter which indicates the filial series. Thus the points marked C, D, H, and J on the curve of the A series indicate the periods when filial series C, D, H, and J were started as ex-conjugants from pairs in the A series.

The successful and negative conjugation tests are shown in diagram 1 by + and - signs, while the inconclusive tests due to lack of material or to adverse conditions under which the tests were made are indicated by question marks. The dotted line connecting the first positive signs in different series indicates that

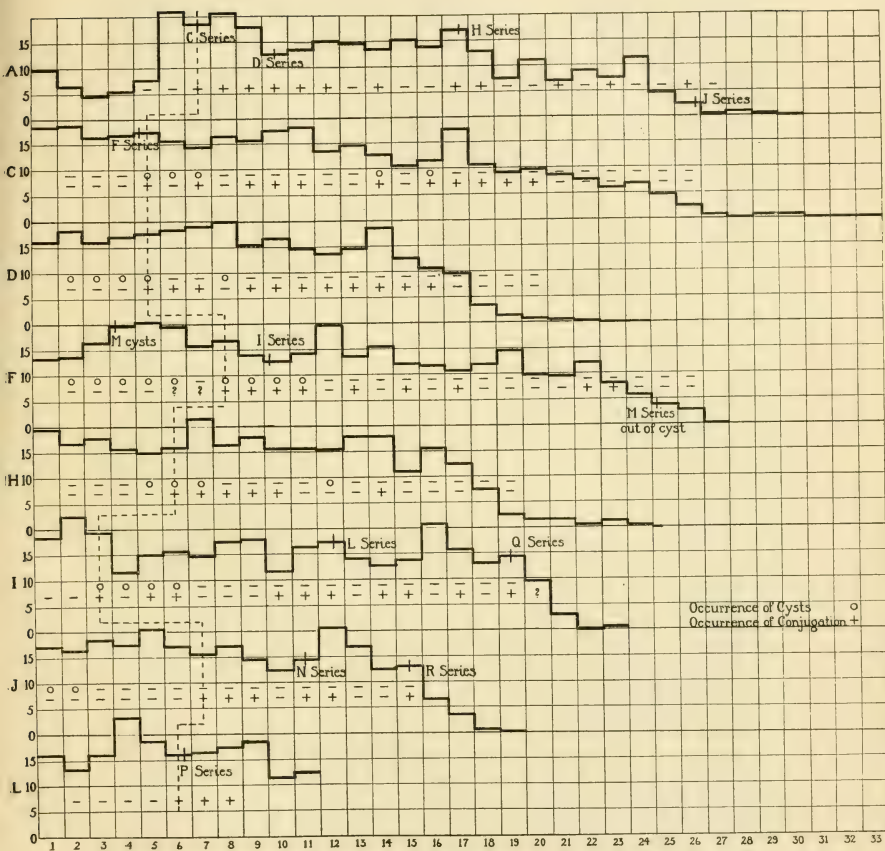


DIAGRAM 1

the protoplasm of each series represented by the curve to the left of this line was in a condition of sexual immaturity. Sexual maturity, or ability to conjugate, persists after the first sixty days practically to the end of the cycle. This is well illustrated by the A series where conjugations occurred regularly up to one month prior to the death of the race. Usually, however, the conjugation tests in the later periods of the cycle are inconclusive, owing to the low division rate and the resulting scarcity of material.

The cysts of *Uroleptus mobilis* are small and spherical and the walls are smooth. When once encysted, the organisms do not emerge from their cyst membranes until weeks afterwards, and then only after an intervening period in a dried condition. Such dried cysts, when placed in fresh culture medium, will give up their contained organisms in from five to twenty days. During such encystment, or prior to it, the protoplasm of the individual undergoes asexual reorganization ('endomixis') and, after emerging, begins its life cycle with an initial vigor similar to that of an ex-conjugant. The B and M series, for example, started from cysts.

Encystments have never occurred in the isolation cultures and the uniformity of the curves is sufficient evidence to show that no other process of parthenogenesis takes place in these cultures. In conjugation tests, however, encystment seems to be due to the same conditions under which conjugation is possible. In such tests the date of the appearance of cysts is always recorded. Such dates are indicated on diagram I by circles above the symbols for the conjugation tests.

As shown in the diagram, encystment usually takes place among some individuals of a conjugation test before the first conjugating individuals appear (series D, F, G, H, and J). In some cases (G and J) encystment occurs in such tests made during the first ten days of the cycle. In other cases (C and I) encystment and conjugation both appeared for the first time in the same test. I have no comment to offer as to the significance of these results, but I hope to get further light on the subject with continued observations.



## RESULTS OF THE EXPERIMENTS TO DATE

The physiological effect of conjugation or fertilization in living things has been interpreted, in the main, along two lines of theory. One of these, and the older, may be indicated by Bütschli's term *Verjüngung* and by Maupas's term *rajeunissement*—terms indicating that the primary effect of conjugation is to restore the lagging vital activities to an optimum. The other theory, first fully elaborated by Weismann, assumes that the union of germ plasms (*amphimixis*), brought about by conjugation, is a source of variation.

These two theories are not reciprocally exclusive, and it is possible that both are correct, although neither has yet been conclusively established.

Bütschli interpreted conjugation in the protozoa as a means whereby waning vitality is restored to full metabolic activity. The problem thus suggested involves three fundamental questions: 1) Does the protoplasm of a single individual protozoon and its progeny by division undergo a progressive waning of vital activities leading to 'old age' and finally to natural death? 2) Does conjugation actually restore such weakening protoplasm to a condition of full metabolic activity? 3) If conjugation accomplishes this, what is the explanation of the result?

The first of these questions has been answered in the affirmative by the experiments of Maupas and of subsequent investigators. The second has never been answered conclusively, although strong experimental evidence has accumulated in support of the affirmative. The third question, obviously, is dependent on the second, and at best can be answered only hypothetically on the basis of our present knowledge.

*1. Does the protoplasm of a single individual Uroleptus mobilis and its progeny by division undergo progressive waning of vitality and natural death if conjugation and parthenogenesis are prevented?*

Table 2 and diagram 1 based upon it show clearly enough that this question is answered in the affirmative. Series A, C, D, F, G, and H, all started as ex-conjugants, show the same initial

vitality and a progressively waning vigor leading to death, while other series, obtained from these by conjugation, fed at the same times on the same standardized culture medium, are now living actively. Counting from the day on which the first division of the ex-conjugant occurred to the day on which the last division occurred, the protoplasm of the A series divided during 267 days; that of the C series during 294 days; the D series, 215 days; the F series, 256 days; the G series, 253 days, and the H series, 245 days. The total number of divisions and the average division rate per individual in each series are shown in table 3.

The C series had the greatest vitality in regard to endurance, while the F series had the greatest vitality in regard to division

TABLE 3

	SERIES					
	A	C	D	F	G	H
Number of division days.....	267	294	215	256	253	245
Number of divisions.....	313	348	271	317	291	268
Average division rate in any ten day period.....	11.72	11.83	12.14	12.38	11.50	10.93

energy. In the latter case the individuals in each line divided on the average 12.38 times in ten days, while in the C series the average was 11.87, and in the H series it fell to 10.93. The difference between F and C is too slight for comment, but that between F and H or between D and H may have some significance in connection with the problem as to whether the offspring vary in vitality according to the age of the parents at the time of conjugation. I have not enough data at present to throw much light on this problem, but the data from which table 3 was derived may be further analyzed to show how the differences between the different series are distributed in the life cycles.

It is shown above that the first conjugations in a series occur, as a rule, from fifty to seventy days after the first division of the ex-conjugant which gives rise to the series. Sixty days, therefore, may be chosen as approximately the period elapsing before the first conjugation in a series, and a period representing the stage

of immaturity of the protoplasm. Adopting sixty days as a unit period, it is possible to work out from the daily records the division rates in all series for the first, second, third, and fourth sixty days of each series to date. These are shown in table 4 in which the mean division rate of a series per day and its probable error have been worked out by Davenport's formulae as shortened by Crampton. For comparison with the preceding tables, these rates are divided by five and multiplied by ten (multiplied by two) to give the number of divisions which each of the five lines of protoplasm of a series is capable of undergoing in ten days. The G series is omitted from this table, as it represented protoplasm which did not come from the original A series. Three other series, I, J, and L, are introduced, although only one period of the last is involved. The mean for the first sixty days of the A series is not included, since this represents the period of experimentation with the culture media at the outset of the work; the standard culture media was first used with this series ten days before the beginning of the second sixty-day period.

The remarkable uniformity of the division rates during the first sixty days in all series regardless of the source, or calendar period, or age of parent, indicates that every ex-conjugant composed of a portion of the original protoplasm derived from A begins its life cycle with a definite optimum division energy indicated by 17.1 to 17.9 divisions per line in ten days. This is the average rate for sixty days, and the rate for the first or second ten day period may be lower or higher than the average for sixty days. Referring to table 2, we find a higher rate than the mean for sixty days in the first ten-day periods of series C, H, and I; while it is lower than the mean for the first sixty days, in the first ten-day periods of series D, F, J, and L. These averages for the first ten days may be even less than the averages for the same calendar periods of the parental series, a fact which furnishes the kind of evidence that has been used by some experimenters as an argument against rejuvenescence by conjugation. Thus in the first ten-day period of the C series, the division rate was 18.6 while that of the parental A series in the same period was 20.8. Again the F series, with



[illegible]



only 13.4 divisions in its first ten-day period, was considerably lower than that of the parent C series, which was 15.6 for the same calendar period. These fluctuations disappear by using the longer, sixty-day period, where in every case the mean division rate of the filial series is greater than that of the parental series for the same period (table 5).

The mean division rates for the second sixty-day periods show a decreased vitality in every series (table 4). Here, again, there is a remarkable uniformity of results, although variations are more marked than in the first sixty-day period. The decrease in the vitality of the H series in this second period is so slight that it would not be noticed were it not for the decrease in all series—a decrease which is especially noticeable in the F and J series.

In the third sixty-day period there is a similar decrease in vitality over the second sixty-day period in all series, and the variations in different series become still greater. Compared with the second sixty-day period, the decrease in vitality is again slight, but compared with the first sixty-day periods, the decrease in vitality becomes sufficiently large to indicate an indisputable waning of vitality in all series.

In the fourth sixty-day periods the vitality shows a marked drop in all series that have passed through this age, and here the variations in different series are extreme. The D series, for example, drops from an average of eleven and a half divisions to seven-tenths of one division in ten days. On the other hand, the F series dropped only from twelve and six-tenths to nine and seven-tenths divisions in ten days.

The precipitous fall in vitality manifested during this fourth period of sixty days is continued into the fifth period. Only a few of the series outlive this sixty-day period, indeed, only the protoplasm of the C series was alive at the end of three hundred days, that of the A series dying during the last week of this fifth period. The decline in vigor and early death were most marked in the F and H series, the former dying four weeks, the latter, three days after the end of the fourth period.

Maupas found that the last individuals of a race were deformed, reduced in size, and degenerate through loss of micronuclei and

cirri. In *Paramecium caudatum* ('04) I found that the micronucleus is degenerated through hypertrophy, while the cortex showed degeneration mainly through the entire absence of trichocysts. Similarly, the last individuals of a series of *Uroleptus mobilis* show evidences of morphological as well as physiological degeneration. They become greatly reduced in size and the micronuclei entirely disappear, probably by absorption. The macronuclei do not disappear, but show characteristic changes indicating degeneration. The eight separate nuclei usually remain independent, but show evidence of an attempt to fuse as they do prior to division in a normal individual. Their chromatin contents, however, are quite different from the normal; instead of an equal distribution of uniform granules, it masses together to form an intensely staining nuclear body similar to those which form from the degenerating macronuclei subsequent to conjugation (fig. 1, 2, 3, 4; cf. fig. 87, and 89, Calkins, '19).

Unlike Maupas's hypotrichs, *Uroleptus* does not show cytoplasmic changes further than reduction in size: the membranelles and cirri do not disappear and the contractile vacuole keeps up its activity.

In this degenerate condition the last individuals of a series are unable to divide—division, apparently, being impossible without a micronucleus. They sometimes show a remarkable tenacity of life, however, in this final phase, indicating that metabolic activities may go on despite degeneration. Thus the last individual of the A series lived for thirteen days after the final (313th) division: the last of the D series for eighteen days; the last of the G series for fourteen days; the last of the H series for fifteen days. The most remarkable cases of longevity of the single individual occurred in the C and the B series. The last individual of the C series lived in its 348th generation for thirty-six days without dividing again, and the last one of the B series, formed as a product of the 257th division on August 31st, lived, without dividing again, until October 9th, a period of forty days. These last individuals, like all others, were transferred daily to fresh standardized culture medium, and their death was not due to violence.

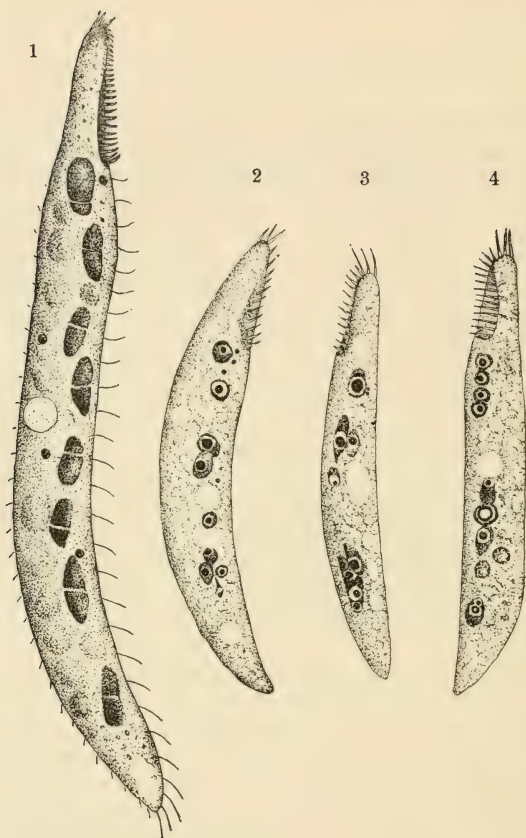


Fig. 1 *Uroleptus mobilis*. Normal individual and individuals degenerated through old age. Camera drawings, same magnification.

1 Normal individual with typical nuclear complex of eight macronuclei and four micronuclei.

2 Individual from the F series in the 315th generation. Four micronuclei still present, but greatly reduced. The macronuclei have undergone granular degeneration. The F series died out in the 317th generation.

3 A prematurely degenerated individual from the C series in the 316th generation. The micronuclei have entirely disappeared. The macronuclei show the characteristic granular degeneration. The C series died out in the 348th generation.

4 Individual from the A series in the 313th generation (a sister cell of the last individual of the series). All of the nuclei have degenerated, the macronuclei by granule formation, the two remaining micronuclei by hypertrophy.

Bearing in mind the fact that, during these periods of declining vigor and death, other filial series derived from their protoplasm through conjugation were living with full vigor, although handled precisely alike and fed at the same times with the same culture medium, we are led to the conviction that waning vitality and natural death are inevitable attributes which are inherent in *Uroleptus* protoplasm, and that the conclusions of Maupas and of subsequent investigators in regard to this phenomenon are fully confirmed.

*2. Does conjugation between two closely related individuals of Uroleptus of the same age result in checking this waning vitality and in restoring the protoplasm to full metabolic activity?*

The conjugation tests are made with individuals of the same age and of the same series, collected on a certain day from the excess individuals after the isolations are made for that day. These excess individuals, sister cells of those continued in the isolation cultures, represent protoplasm the history of which is identical with that of the protoplasm of the isolation cultures. They are placed in a Syracuse dish with an abundance of culture medium. Here they multiply, and their progeny, in the course of two or three weeks, will conjugate, provided they are sexually mature.

One such pair of conjugating individuals is isolated in fresh culture medium. The two individuals will have separated as ex-conjugants in twenty-four to thirty-six hours, and one of these is isolated to start a filial series. Thirteen such filial series have been started; five of these have completed their full cycles and have died out; eight, in various stages of vitality, are still under observation.

The question above has already been answered positively by the results shown in table 4. Every ex-conjugant from the A protoplasm, regardless of the phase of vitality in which the conjugation occurred, shows an average division rate during the first sixty days of life of 17.1 to 17.9 divisions in ten days. Table 4 also shows that this is the highest average division rate of all sixty-day periods of the life cycle. Environmental conditions of food, treatment, etc., being the same for parental and filial

series, the only possible explanation of the restoration of vitality in filial series lies in the process of conjugation by which such filial series are started.

Although table 4 shows that all ex-conjugants have practically the same optimum division rate at corresponding periods of the life cycle, it does not show the actual differences in metabolic activity between parent and filial series in identical calendar periods. If conjugation does not restore vitality, the division rate of a filial series should not differ from the division rate of the parent series from which it has been derived; on the other hand, if it does restore vitality, the extent to which it is restored will be indicated by the difference in the average division rates of parental and filial series in identical calendar periods.

These differences in average division rates of parent and offspring series are clearly shown in table 5. Here two interesting phenomena are evident: first, reading across, the table shows that the discrepancy between parent and offspring increases with the age of the parent series at the time of conjugation; second, reading down, it shows that, while both series are losing vitality, the parent series is losing it more rapidly than the filial series.

In regard to the first of these phenomena, it will be noted that the C series came from the A series when the latter was in the 78th generation and that, during the first sixty days of the filial series, the protoplasm of each line had the power to divide 1.53 times more in ten days than that of each line of the parent A series in the same period. The D series came from the A series when the latter was in the 137th generation, that is approximately fifty generations older than the protoplasm which gave rise to the C series. The discrepancy now between the parent series A and the filial series D amounted to 3.03 divisions in ten days during the first sixty days of the D series. That is the protoplasm of each line of the offspring series had the power to divide 3.03 times more in ten days than that of each line of the parent A series. The filial H series came from the same parent A series when the latter was in the 237th generation, or about 150 generations older than when the C series was formed. The discrepancy between parent A and offspring H series now



amounted to 4.8 divisions in ten days. Finally, the J series came from the same parent A series when the latter was in the 311th generation, i.e., the protoplasm was about 230 generations by division older than it was at the time when the C series came off. The discrepancy in division rates between the young J series and the old parental A series now amounted to 17.6 divisions in ten days. This means that, if the protoplasm contained in the two weakened cells of the parental A series had not conjugated in the 311th generation, it might have divided during the subsequent sixty days at the rate of twenty-five hundredths of one division in ten days, but having conjugated, it actually divided at the rate of 17.9 times in ten days.

These results indicate that the protoplasm of *Uroleptus* under these cultural conditions has a certain optimum capacity or potential of metabolic activity which is gradually exhausted, but which can be restored by conjugation. The greater the exhaustion, the more remarkable the restoration. As recharging a storage battery restores its potential of active energy, so conjugation restores the potential of vital energy. If the battery is recharged before the old charge has been drawn upon, its potential of activity would scarcely be affected. An analogous condition is shown by the *Uroleptus* protoplasm after encystment, and after conjugation occurring in an earlier period of the life cycle (e.g., the A and C series, or the C and F series). If, however, the battery is nearly exhausted before it is recharged, the difference in potential between the newly changed condition and the exhausted condition would be marked. The analogue to this is the J series and the parental A series. The restored potential of vitality in *Uroleptus* protoplasm is a 'charge' of vital energy which is capable of metabolic activities through a period of from 260 to 300 days, or vitality sufficient to produce individuals to the number of 2 to the 300th + power.

In regard to the second of the phenomena shown by table 5, it is interesting to see that, as the protoplasm of a series grows older, the potential of vitality is exhausted at an increasingly rapid rate. Thus, the discrepancy between the division rates of parental and filial series is greater during the second sixty-day

TABLE 5  
*Comparative vitality of parents and offspring*

	C FROM A SERIES	D FROM A SERIES	H FROM A SERIES	J FROM A SERIES	F FROM C SERIES	I FROM F SERIES
AGE OF PARENT.....	78 generations	137 generations	237 generations	311 generations	86 generations	143 generations
Dates	2/7 to 4/8	3/8 to 5/7	5/22 to 7/21	8/16 to 10/15	3/28 to 5/27	7/7 to 9/5
Mean daily divi- sion rate all five lines	8.6333 7.8667	8.5833 7.0666	8.6666 6.2666	8.9666 0.125	8.6000 8.1000	8.5833 6.5666
Average division rate per line for 10 days	17.2666 15.7334	17.1666 14.1333	17.3332 12.5332	17.9332 0.2500	17.2000 16.2000	17.1666 13.1332
Increase per line of filial series in each 10 days	1.5332	3.0333	4.8000	17.6832	1.0000	4.0334
Dates	4/8 to 6/7	5/7 to 7/6	7/21 to 9/19		5/27 to 7/26	9/5 to 11/4
Mean daily divi- sion rate all five lines	7.9167 6.6000	8.1666 5.2833	8.5333 1.5500	Parent dead.	7.7834 6.6667	7.9834 5.6666
Average division rate per line for 10 days	15.8333 13.2000	16.3333 10.5000	17.0666 3.1000		15.5668 13.3334	15.9668 11.3332
Increase per line of filial series in each 10 days.	2.6333	5.7733	13.9666		2.2334	4.6336

First 60  
days of  
offspring.

Second 60  
days

Third 60 days	Dates	6/7 to 8/6		7/6 to 9/4		9/19 to 11/18		7/26 to 9/24		11/4 to 1/3	
	Mean division rate all lines	6.4166	4.3650	5.7834	2.234	Parent dead		6.3333	4.0166	7.5166	2.0800
	Average division rate per line for 10 days	12.8332	8.7300	11.5668	4.4680			12.6666	8.0332	15.0333	4.1600
	Increase per line of filial series in each 10 days.	4.1032		7.0988				4.6334		10.8733	
Fourth 60 days	Dates	9/24 to 11/23		9/24 to 11/23		9/24 to 11/23		9/24 to 11/23		9/24 to 11/23	
	Mean daily division rate all five lines	Parent	dead	Parent	dead			4.8833	1.2500	Parent	t dead
	Average division rate per line for 10 days							9.7666	2.5000		
	Increase per line of filial series in each 10 days.							7.2666			

period of the filial series than during the first sixty days. Indeed the discrepancy between parent and offspring in successive sixty-day periods increases in geometrical proportion. Thus the differences between the A and C series for the first, second, and third sixty-day periods are indicated by 1.53, 2.63, and 4.10 divisions in ten days. The differences in the A-D series are 3.03, 5.77, and 7.09. In the C-F series, the differences for four successive sixty-day periods are 1.0, 2.23, 4.63, and 7.26, which is a geometrical increase of the disparity in division energy of parent and offspring protoplasm.

When we consider that we are dealing here with one protoplasm derived from the protoplasm of the single individual ex-conjugant that was isolated on November 16, 1917, these results offer conclusive evidence that conjugation rejuvenates or restores vitality to an optimum when that vitality is reduced through continued metabolic activity. Many series have died, but I have under cultivation to-day protoplasm of the L, N, P, O, and R series which is directly descended from the original ex-conjugant A and which is living with the same metabolic vigor as that shown by the A series during its most vigorous period. Yet there has been no change in the standardized culture medium with which this protoplasm has been fed, and no variation in the daily treatment. The continued vitality is due solely to the successive conjugations which have taken place between representative bits of this protoplasm.

Since one condition, viz., starvation, is found in the conjugation tests and not in the isolation cultures, the objection might be raised that changes may be set up in the protoplasm due to such starvation or to some other condition of the conjugation tests which would result in a restoration of vitality, thus making conjugation an accessory phenomenon without effect on rejuvenescence. To test this point twenty individuals which had reached this starvation point as shown by reduced size, and all taken from a conjugation test, but without having conjugated, were isolated and carried on in isolation cultures as though they were ex-conjugants. An ex-conjugant from the same test, and obtained from a pair that were isolated while conjugating, was likewise carried on at the same time in five lines as series U.

The results (Table 6) show that conditions of the conjugation test have no stimulating effect on the protoplasmic activities. Indeed there is evidence of a depressing effect as indicated by the average division rates for sixty days. The rates for non-conjugants are not only lower than that for the ex-conjugant (U Series) but, in all cases, are lower than that of the control parental series. All were descendants of the same series (L series) and all were of the same age. The starved individuals are grouped in four aggregates of five each in table 6, as follows:

TABLE 6

	PARENT RACE L SERIES (5 LINES)	EX-CONJUGANT FROM L SERIES U SERIES (5 LINES)	NON-CONJUGANTS FROM SAME CONJUGATION TEST AS U SERIES. TWENTY INDIVIDUALS IN 4 GROUPS OF 5 EACH			
			Group 1	Group 2	Group 3	Group 4
Average division-rate first 10 days.....	8.4	9.2	7.0	7.6	7.4	6.8
Second 10 days.....	5.4	14.0	5.8	6.6	6.6	6.8
Third 10 days.....	10.0	13.4	4.4	5.6	6.8	5.4
Fourth 10 days.....	10.4	14.0	7.0	6.6	8.4	5.4
Fifth 10 days.....	7.6	13.6	6.6	6.8	7.2	7.2
Sixth 10 days.....	5.6	13.6	7.8	7.6	6.4	7.4
Average division-rate 60 days.....	7.9	12.96	6.4	6.8	7.1	6.5

There is some evidence, by no means complete as yet, that vitality of the more recent series lacks the endurance of the earlier series. This is apparent in diagram 1, where the curves of the filial series are progressively shorter from the C series to the J series. Both I and J are now in the last stages of metabolic vigor and no further divisions in either series will occur. The I series is now 235 days old, and the J series 200 days, the former having divided 322 times, the latter 253 times. The shortened cycle appears to be accompanied by greater intensity of division energy than in the earlier filial series, the I series having an average division rate of 13.2 divisions in ten days, the J series, 12.6 (table 3). In a few months the cycles of the L, N, O, P, and R series will be complete and will furnish more adequate data for conclusions on this interesting point.



The Q series, coming from the I series in the 316th generation, has been queer from the start, dividing only twenty-three times in thirty-three days, and it will soon die out. The exceptional history of this series is probably due to faulty reorganization after conjugation, for the nuclear complex is quite abnormal. If this result is due to the age of the parent series at the time of conjugation, the history of the Q series is an interesting contradiction to that of the J series where the parent series was relatively even older at the time of conjugation. Defective reorganization is possible after any conjugation, but the chances of such defective reorganization are probably greater with increasing age of the parent.

While the several series described above were all derived from one ancestral protoplasm of the A series, two other series, B and G, came from a different source. The B series was started from an encysted *Uroleptus mobilis* which had encysted in 'wild' stock before the A series was started. It emerged from the cyst on January 25, 1918, and lived until October 9th, dividing 258 times between January 25th and September 1st, or 11.8 divisions in ten days on the average. Many epidemics of conjugation occurred, but only one ex-conjugant was isolated. This one formed the G series, the B series being in the 115th generation at the time. The same resultant renewal of vitality and continued life of the filial series was observed as with the A protoplasm, the G series starting with an optimum division rate of 18.06 in ten days for the first sixty days, running through 291 generations and dying out January 4, 1919.

*3. Does reorganization during encystment ('endomixis' or parthenogenesis) restore waning vitality to full metabolic vigor?*

Unfortunately, I have insufficient data to draw positive conclusions on this subject. Only two series, B and M, were derived from cysts, and of these only the M series is pedigreed. This series was started on November 18th from a cyst that was formed by an individual of the F series in its 45th generation, on April 27, 1918, and remained encysted for six months. In

the first period of sixty days after emerging from the cyst, it had a higher division rate than any other representative of the A protoplasm, the protoplasm of each line dividing, on the average, 19.8 times in ten days. If the vitality of the encysted individual was the same in potential as that of the same protoplasm in the isolation cultures, we would expect the division rate of the isolation series during the sixty days subsequent to the 45th generation to be practically the same as that of the protoplasm from the cyst. This expectation, however, was not realized, for during this period the F series divided only 17.6 times in ten days—a difference in potential of 2.2 divisions in ten days. The difference between the division rate of the F series for its first sixty days and the M series for its first sixty days was 2.6 divisions in ten days (table 4).

The difference between the division rates of the parent F series and the offspring M series by parthenogenesis is not actually as large as the figures indicate. The M series was maintained under the conditions of a constant temperature of 24°C., while the F series was maintained under the conditions of laboratory temperature which varied from 19° to 22°C. That the difference in rate is not due solely to these different conditions, however, is shown by a comparison of the M series with the L series (cf. Table 2). The latter came from the I series and the I series from the F series. Both L and M, therefore, had the same ancestry. Furthermore, L and M were started at approximately the same time, L as an ex-conjugant, M from a cyst, and both series were maintained under the same temperature conditions. The L series, during the same sixty days as above for the M series, had an average division rate of 18.8 in ten days as against 19.8 for the M series.

The unpedigreed B series, which came from a cyst, may be compared with the C series which started as an ex-conjugant at about the same time as the B series. The first sixty days of the B series gave an average division rate of 17.4 divisions in ten days, while that of the C series was 17.2.

So far as the evidence thus far obtained is concerned, it appears that the initial vitality after encystment and partheno-

genesis is as great as, or even greater than, that after conjugation. It remains to be seen whether this high potential has the same capacity of endurance as that obtained from conjugation. The M series is not old enough at the present time to furnish evidence. The B series divided only 258 times, while its contemporaries, the A and C series, divided 313 and 348 times, respectively.

#### GENERAL

It is not my intention to formulate here any theory in explanation of the phenomenon of rejuvenescence. The facts concerning it may be grouped in two categories: one, physiological, the other, morphological.

In the first place, the results presented in this paper show that in *Uroleptus mobilis* the physiological processes of metabolism are not capable of unlimited activity. The limits vary from the time of conjugation or encystment to between 268 (H series) and 349 (C series) generations by division. Within these limits there is a progressive weakening of metabolic vigor from an optimum shown during the first three months after conjugation. This weakening, furthermore, increases by geometrical progression, i.e., it is cumulative, as shown by the geometrical increase of the difference in vitality between filial and parental series in successive sixty-day periods (table 5). Such weakening protoplasm, if not allowed to conjugate, inevitably dies, as does the somatic protoplasm of metazoa.

A second physiological fact is equally well established by these *Uroleptus* experiments. The same protoplasm is transformed from a condition of metabolic weakness to a condition of optimum metabolic vigor by the process of conjugation. The effect of conjugation is clearly indicated by the extreme case of the J series. Here the protoplasm, at the time of conjugation, had only enough metabolic vigor to divide twice in ten days. The cells that conjugated were both composed of this weak protoplasm. Ten days later if they had not conjugated, each might have been able to divide at the rate of 0.25 times in ten days, or once in forty days; but, having conjugated, one of them, the

J series, was able to divide at the rate of 17.9 times in ten days, or 71.6 times in forty days, and there is no reason to doubt that the other ex-conjugant of this pair would have had the same vigor.

The experiments also indicate that there is a limit to the extent to which this protoplasm can be rejuvenated. It might be inferred, with reason, that if two weak individuals are transformed by conjugation into individuals capable of dividing seventeen times in ten days, then conjugation between two individuals from a series in which physiological weakness is not yet perceptible, would result in an ex-conjugant capable of dividing more than seventeen times in ten days. The inference, however, is not supported by the facts. A good illustration is the relation between the F and C series (cf. Table 5). The F series came from C when the latter's vitality was indicated by 17.2 divisions in ten days. Each of the two individuals conjugating at this time, would have had the ability to divide at the rate of 16.2 times in ten days during the ensuing sixty days, if they had not conjugated. Having conjugated, they were able to divide at the rate of 17.2 times—a difference, or extent of rejuvenescence, indicated by only one division in ten days. Since all ex-conjugants, under the conditions of the experiments and regardless of the state of vitality of the parent protoplasm, return to this same optimum of vitality measured by 17+ divisions in ten days (table 4), it is evident that the protoplasm of *Uroleptus* will hold only a certain charge, so to speak, or potential of metabolic vigor, as a result of conjugation. This optimum, of course, is subject to change by changes in the environmental conditions—heat, for example, increasing it.

A third physiological fact is also indicated by these experiments, although the numerical support is not as adequate as that in support of rejuvenescence through conjugation. This is the fact of rejuvenescence following encystment and parthenogenesis, in which no nuclear interchange occurs. The B series and the M series came from cysts. The ancestry of the former is unknown, but during its first sixty days in culture, its metabolic vigor was measured by a division rate of 17.4 times in ten days.

This is the same optimum as that following conjugation. The M series is pedigreed. It came from the F series in its 45th generation, and after a period of six and a half months was recovered from the cyst. During the sixty days subsequent to the date of the 45th generation, the F series divided at the rate of 17.6 times in ten days, while the protoplasm of the M series during its first sixty days of culture divided at the rate of 19.8 times in ten days. The difference (2.2 divisions) is undoubtedly greater than it would have been had the temperature conditions remained the same. As explained on page 151, the M series was cultivated under conditions of higher temperature than the laboratory, whereas the F series was cultivated under the laboratory temperature. The L series, which came from the F series through conjugations and which was started at approximately the same time as the M series, was cultivated under the same conditions as the M series. Its division rate during this same period of sixty days was 18.8—one full division higher than the usual ex-conjugant. In this case of the M series, therefore, the rejuvenating effect of parthenogenesis was even greater than that of conjugation. Whether the endurance of the parthenogenetic protoplasm differs from that of the protoplasm following conjugation remains to be seen.

Parthenogenesis through encystment appears to be an attribute of high vitality, and the ability to encyst is apparently lost at an early date (diagram 1). In the C series it did not occur after the 160th day; in the F series, not after the 110th day, and in the D, I, and J series it did not occur after the 80th, 60th and 20th days, respectively. I do not know what this means, but it is certainly true that no internal reorganization without encystment has occurred thus far, for in every series the physiological depression is continuous and progressive, and death invariably follows. Conjugation, with rejuvenescence, however, is possible almost to the end of the cycle. Encystment, apparently, is not possible near the end of the cycle, but it does occur even in the first ten days after conjugation. Conjugation, on the other hand, does not occur until from thirty to seventy days after the previous conjugation. I am aware of published state-



ments to the contrary in connection with other ciliates, and it may well be that this condition of sexual immaturity noticeable in *Uroleptus* is not universal among ciliates.

As Maupas found for other hypotrichous ciliates, the condition of physiological depression is accompanied by morphological changes. During the period of active metabolism the cell rapidly grows to full size after division ( $140\ \mu$  to  $165\ \mu$ ). The macronuclei are eight in number, with from four to six micronuclei. In preparation for division a portion of each macronucleus is thrown off and is absorbed in the cytoplasm, while the remaining portions fuse to form a single division nucleus. All but two of the micronuclei are likewise absorbed. In the late individuals of a cycle, the macronuclei lose their characteristic nuclear clefts and in some cases show a tendency to fuse, while in other cases the number is increased from eight to as many as sixteen smaller and irregularly shaped nuclei. The micronuclei do not increase in number, but undergo degeneration by hypertrophy or by granular degeneration, and finally disappear in the cytoplasm. In the last individuals of a series, the chromatin of each macronucleus collects in a single large, highly refractile, and densely staining granule. The size of the cell is greatly reduced and it is unable to divide. The cytoplasm is probably as much changed as the nuclei, but morphological evidence of such change is difficult to detect. In general there is a tendency to increased vacuolization, while the mitochondria, which form a cortical layer in the normal individual, are rare and irregularly distributed. Some individuals in this final stage of depression live without dividing for thirty-six (C series) and forty (B series) days.

In conjugation, apart from the processes of maturation and reduction in number of chromosomes from eight to four, and union of the gametic nuclei, the most significant phenomenon is the granular disintegration of the old macronuclei, and absorption of the relatively large quantity of nuclear substance in the cytoplasm. Not only do the macronuclei thus furnish nucleoproteins to the cytoplasm, but the micronuclei also contribute no small part. Thus, if an individual goes into conjugation with

four micronuclei, all four of them may undergo the first maturation division. Of the eight possible micronuclei thus formed, only two undergo the second maturation division, while six are absorbed. All of the four products of the second maturation division may undergo the third division, and of the eight products of this phase, only two become pronuclei, the other six being absorbed. Finally, in the second division of the amphinucleus, two of the four products form the new micronuclei, one forms the new macronucleus, while one, the sister nucleus of the new macronucleus, is absorbed in the cytoplasm (Calkins, *loc. cit.*, pp. 316-326).

The cytological details of encystment are not yet worked out. Anticipating the description of the process, it may be briefly stated here that the macronuclei break up into granules as they do after conjugation, and these granules are absorbed in the cytoplasm.

One phenomenon, therefore, common to division, conjugation, and encystment, is the absorption of variable quantities of nuclear substance in the cytoplasm. That the physical and chemical consequences of such absorption are connected with the phenomenon of rejuvenescence seems probable. That the relation between the new amphinucleus after conjugation, or the new nuclear complex after encystment, and this reorganized cytoplasm is likewise connected with the phenomenon of rejuvenescence is equally probable. The nature of such connections and of such relations is a matter of speculation for which we are not yet prepared.

Columbia University

March 4, 1919



Resumido por los autores, Leslie B. Arey y W. J. Crozier.

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### Las respuestas sensoriales de Chiton.

En el presente trabajo los autores pasan en revista las capacidades sensoriales y modos de reacción que presenta *Chiton tuberculatus* Linn., con referencia particular a la diferenciación de los receptores y a la significación etiológica de ciertos tipos de respuestas. Bajo el primer epígrafe señalan que se puede demostrar un grado considerable de diferenciación sensorial, y bajo el segundo prestan atención a la conexión entre las modificaciones progresivas del heliotropismo en relación con la edad, el modo en que se determinan estos cambios y sus consecuencias, que aparecen en la vida de una población de *Chiton*. La bionómica de *Chiton* descubre una serie intrincada de interrelaciones armónicas, necesitandose una descripción exacta de su historia natural. Los autores señalan el hecho de que el medio ambiente en que vive este molusco "primitivo," en el cual la centralización nerviosa se presenta en un estado incipiente, está determinado en varias edades por el comportamiento del animal, con ciertas relaciones ventajosas realizadas de una manera automática.

Translation by José F. Nonidez  
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# THE SENSORY RESPONSES OF CHITON<sup>1</sup>

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FOURTEEN FIGURES

## CONTENTS

I. Introduction.....	158
II. Natural history.....	160
1. Habitat and appearance.....	160
2. Growth and duration of life.....	169
3. Destructive agents.....	170
4. Feeding.....	172
5. Respiration.....	173
6. Migrations; association in groups.....	175
7. Breeding habits.....	179
8. Bionomic correlations.....	181
III. Movements and reactions.....	182
1. Local movements.....	183
2. Movements of the animal as a whole.....	185
3. Locomotion.....	187
IV. Mechanical excitation.....	192
1. Tactile stimulation.....	192
A. Dorsal surface.....	193
B. Ventral parts.....	194
C. Distribution of sensitivity.....	198
D. Tactile receptors.....	200
2. Vibratory stimuli.....	202
3. Thigmotaxis.....	204
4. Rheotropism.....	206
5. Geotropism.....	207
6. Summary.....	210

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<sup>1</sup> Contributions from the Bermuda Biological Station for Research, no. 110, and from the Anatomical Laboratory of the Northwestern University Medical School, no. 66.



V. Thermal excitation.....	213
1. Behavior at different temperatures.....	213
2. Local application of heat and cold.....	221
3. Summary.....	223
VI. Photic excitation.....	224
1. Effects of light.....	224
a. Behavior in an illuminated field.....	224
b. Results of partial illumination of the body.....	228
2. Differential sensitivity.....	230
a. Shading.....	230
b. Increased light intensity.....	232
3. Distribution and nature of photoreceptors.....	233
a. For illumination.....	233
b. For shading.....	234
c. For increased illumination.....	235
4. On the theory of phototropism.....	235
a. The orienting stimulus.....	235
b. The determination of positive and negative orientation....	236
c. The method of orientation.....	237
5. Bionomic correlations involving photic behavior.....	238
VII. Chemical excitation.....	240
1. Reactions to various substances.....	240
2. The mode of excitation by solutions.....	246
3. The chemoreceptors.....	250
VIII. The nervous system and sense organs of Chiton.....	254

## I. INTRODUCTION<sup>2</sup>

Chitons of the species *C. tuberculatus*<sup>3</sup> constitute, in appropriate situations, a conspicuous element in the shore fauna of the Bermuda Islands. Their large size, their abundance, and the fact that when mature the sexes are readily distinguishable by external inspection (Crozier, '19) make these chitons favorable animals

<sup>2</sup> Many of the experimental observations used in this report were originally obtained by L. B. A. during the summer of 1914, and were briefly reported at the fifteenth meeting of the Zoological Society (Arey, '18 a); some phases of this inquiry have since then been worked over independently by W. J. C., who has added matter drawn from field studies and from further experimentation, and is responsible for the actual writing of the paper. In 1914 our work was made possible by grants from the Humboldt Fund of the Museum of Comparative Zoölogy; we would express our appreciation of this support.

<sup>3</sup> *Chiton squamosus* L. was obtained by the Challenger expedition near Bermuda (Haddon, '86), and this is the only species listed by the early conchologists for the Bermuda area (e.g., Jones, '88). Heilprin ('89, p. 176) speaks in addition of '*C. marmoratus* Gmel.' and Verrill ('02, p. 497) makes incidental mention of '*C. marmoreus*.' Undoubtedly, the '*marmoreus*' is intended to refer to the

for a variety of investigational purposes. Little has been known, however, about the activities of placophorans, and no experimental study has hitherto been made as to the nature of their somewhat remarkable sensory organs. In the following pages we shall be concerned with the behavior of *C. tuberculatus* and with the several ways in which its responses to sensory activation may be regarded as of general theoretic interest.

Although it would be unwise to place upon the fact too great an emphasis, in relation to the present study, it is nevertheless well to recall that the Placophora are usually admitted to rank as 'generalized,' or even 'primitive,' mollusks. In their marked bilateral symmetry, their somewhat diagrammatic organization, and the primitive aspect of their development (Heath, '99), the Placophora are believed to display features of an ancient and generalized character—which is not, however, fully supported by the facts of their known fossil record, although animals clearly of the chiton type are known from later Ordovician times onward. We need not be led to expect that the primitive organization of the chitons should necessarily determine the relative complexity of their behavior as contrasted with that of other mollusks. The very fact of their greater antiquity, apparently confirmed by the morphological evidence, allows all the more opportunity for the possible acquisition, among the chitons, of special features of their own. From this standpoint little can be gained through the deliberate consideration of the behavior of Chiton in the light of its morphological approximation to the 'ancestral mollusk;' on

animal with which we have worked. In superficial characters it agrees with the usual diagnosis of *C. tuberculatus* (Dall and Simpson, '01). with which *C. squamosis* was for a long time considered to be identical. Specimens have been identified for us as *C. tuberculatus* by Mr. W. F. Clapp, of the Museum of Comparative Zoölogy, to whom we are indebted for this and numerous other kindnesses. In external appearance this chiton is very variable, but it is undoubtedly the only species at all common at Bermuda. In three years' collecting by one of us (W. J. C.) only a single *Acanthopleura* has been seen, although *Ischnochiton purpurascens* is fairly abundant, and *Acanthochites* and *Tonicia* less so. Thus the chiton fauna of Bermuda differs much from that of the more southern portions of the West Indian faunal region, where *Acanthopleura* is said to be the commonest type, although four or five species of chiton proper, including *C. tuberculatus*, are well known from Porto Rico and other stations (W. J. C.).

the other hand, the peculiar disposition of the nervous system in placophorans may allow us to analyze a few generalized, or fundamental, characteristics of the molluskan nervous system. To this end, some acquaintance with the more obvious features of the natural history of *Chiton* and a knowledge of the variety of its motor responses are essential preliminaries.

## II. NATURAL HISTORY

### 1. *Habitat and appearance*

Numbers of *C. tuberculatus* may almost always be discovered in any intertidal situation where the substratum is hard and firm, not too greatly exposed to wind and waves, free from muddy silt, the water not too stagnant nor the growth of algae too vigorous. The diversity of local habitats includes: freely exposed faces of cliffed shores, pockets in shore rocks, crevices, the floors of caves with northern or southern exposures, the under surfaces of boulders and slabs of stone, and the under sides of smaller stones on sheltered, shingly beaches. The individuals obtained from these different habitats are not of uniform appearance. The external aspect of the chitons—as determined by their size, their coloration, and the organisms dwelling upon their dorsal surface—is closely correlated with the conditions under which they are individually found to be living. The meaning of this correlation we are not primarily concerned to analyze in this report; but since the degree of sensory differentiation which we have discovered in *Chiton* and the complexity of its behavior seem in some ways to be exceptionally great, as compared with what is known for some other invertebrates, it is necessary to outline a few of the more important features in its life-history. On certain points the statements made are of a preliminary kind, and must for the present remain in dogmatic isolation. Materials for a quantitative analysis of the bionomics of *Chiton* will be made the subject of a separate report.

The animals used in our experiments were collected in the region of Great Sound. Within this portion of the Bermuda area, to which the application of the following remarks is to be

restricted, *C. tuberculatus* is relatively abundant. During late summer and autumn, young individuals, less than 1 cm. in length, are to be found beneath low-tide level on the under surfaces of stones, glass bottles, and other more or less smooth objects. In spring and summer the youngest (smallest) specimens obtainable are characteristically found under small, flat stones on sheltered beaches, at the upper limit of the tides. Larger individuals are not usually found in this type of habitat. The age at which sexual maturity comes about (Crozier, '19), namely, when a length of about 3.5 cm. is attained, is correlated in a general way with the occurrence of the chitons further down the shore, under larger stones or in crevices in the walls of caves. Still larger chitons, 5 to 7 cm. long, commonly occur freely exposed upon sunlit rocks, while those of maximal size (8 to 9 cm.), are rarely found concealed in dark places.

The youngest chitons, which as a rule live under small stones and are seldom if at all found exposed to bright sunlight, are of a general light greenish cast. The periostracum is lustrous and perfectly intact (uneroded). Very rarely indeed are adventitious organisms (barnacles, *Spirorbis*, or algae) found lodged upon them. In many, in fact in most, places their coloration matches decidedly the tint of their surroundings among the smooth, greenish under surfaces of the stones occurring at the upper reach of the tide. Moreover, the pigmentation is not 'solid,' but is broken up by small light and dark blotches. The homochromicity of the pigmentation is not, however, in all cases perfect.

In coloration and general appearance *C. tuberculatus* assumes the aspect more characteristic of the species at a slightly later period. Animals 5 to 7 cm. long (fig. 1) correspond in form and markings with Haller's figure of *C. squamosus*, well known through its reproduction in many zoölogical texts. The close relationship of *C. tuberculatus* and *C. squamosus*—which for our purposes is fortunate because the anatomy of the latter and of its relatives is well understood—is shown by the fact that until comparatively recent times these species were considered identical by conchologists. At least two distinct pigment materials, and perhaps a third, enter into the general coloration of *C. tuber-*

culatus. The predominating tint of the periostracum upon the valves is green, but in the depressions between the ridges upon both jugum and pleura a brown substance is evident. The

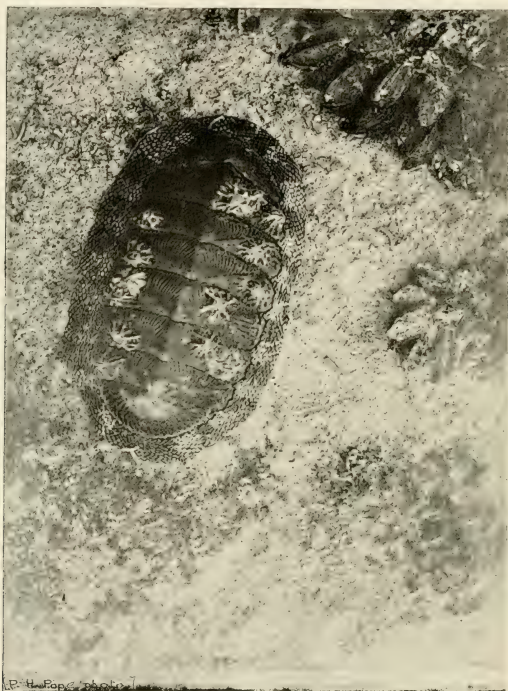


Fig. 1 Photograph of a *Chiton tuberculatus* L. on natural rock substratum, from a moderately protected station, at low tide; about six years old, natural size. Note epizoic barnacles (*Tetraclita porosa* Dar.), also, in the upper right corner of the photograph, a cluster of *Modiolus*, and, on the posterior margin of the girdle, two fecal masses.

tubercles on the pleurae, in specimens less than 3 cm. long, are tipped with blue. In very young chitons (less than 0.9 cm.) the 'scales' covering the girdle are colored opposite the anterior margin



of each shell plate with the same blue-green pigment; the colored scales are disposed in such a way as to form distinct, transverse bands. Usually at a length of about 2.5 cm. this blue-green hue upon the girdle bands is replaced by one of deep brown (burnt umber); at a length of 3.5 cm. the girdle bands have usually begun to fuse at the periphery, although the pale whitish patches of 'scales' lying between them may persist until an advanced age. This has the effect of breaking the solidity of the girdle outline, when it is looked at from a little distance.<sup>4</sup> Whatever concealing effect might be attributed by some writers to the coloration of the girdle in Chiton is completely defeated, however, by one important fact: the chitons found in situations where this condition might be of some use in the way of concealment have the central area of each valve eroded (periostracum and superficial layer of the tegmentum removed), producing along the lateral region of each pleuron an isolated area, which, less eroded, affords a better foothold for algae; both when exposed to the air and when under water the succession of these uneroded areas produces the effect of a solid, dark band, about the width of the girdle, completely outlining the margin of the shell.

The modes of pigmentation, involving blue-green and brown hues, to which we have referred, are generally exhibited throughout the genus Chiton. The green coloration in particular is not, however, solely a matter of pigmentation. In early life the green material is confined to the periostracum. Its intensity varies with habitat, and ranges from a gray green to deep olive green. At lengths above 3.5 cm., however, the chiton becomes covered with a thin coating of dark green algae. The character and extent of this coating varies with habitat. In some cases the green pigment may completely disappear, producing chitons of the 'variety' *assimilis* of Reeve. In all cases there is a pronounced homochromic element in the general appearance of the chitons.

<sup>4</sup> A small species of *Onchidella* found in this habitat is (like many other *Onchidellas*) also pigmented according to this general plan. The free margin of its mantle is colored with a dark brown pigment, interrupted at regular intervals by light patches containing glands. The dark pigment forms denser, but irregular, patches upon the dorsum; the whole effect is of a dense, but moderately homochromic, oval mass surrounded by a banded 'girdle'—but all on a very minute scale, as the creature is only 2.5 mm. long.

The further development of this homochromic characteristic is conditioned by, or goes together with, the erosion of the shell valves. This becomes noticeable at the age of five to six years, commonly, and may be exhibited in a non-subjective manner, as follows:

If the length of the tegumentum along the middorsal line of the fourth valve be measured for a series of chitons from any one locality, and plotted against the length (or the estimated age) of the corresponding individuals, a graph is obtained (fig. 2) show-

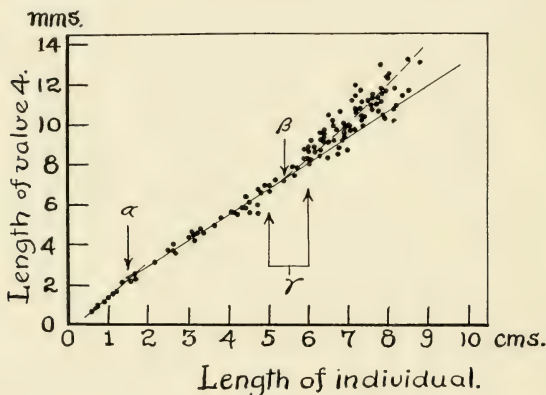


Fig. 2 Showing the relation between length of the fourth valve, in millimeters, and the total length of the individual in centimeters, for chitons of a range of sizes. (Marshall Island, south side, May 9, 1918.) See text and fig. 3;  $\alpha$ , point at which 'beak' begins to project;  $\beta$ , 'erosion point,' estimated from the curve;  $\gamma$ , range of individuals in which (on inspection) erosion was judged to be just beginning.

ing one point ( $\alpha$ ) of sharp bending, at an early age, followed at a more advanced age by a slower change in curvature ( $\beta$ ), initiating a region of less decided correlation between valve length and length of individual. These changes in the course of the graph originate in the following way: In very young chitons the beak ('umbo') on the fourth valve is quite undeveloped, and does not

project beyond the posterior margin of the valve. When the chiton reaches a length of 1.5 cm. the beak begins to project, when 5 and 6 cm. in length, the umbo of the preceding valve (third), and of the other valves, becomes noticeably eroded. Correlated with the incipient exposure of the intertegmental mantel between valves three and four—produced by the erosion of the beak of valve three—there occurs a growth of the anterior edge of valve four, forming a protecting projection into the laminal sinus of this valve, which compensates for the wearing down of the beak on valve three. These relations are illustrated in figure 3.<sup>5</sup> We do not consider here the fine question as to whether this compensating growth of the tegmentum along the anterior margin of the valve is a normal growth process, independent of erosion. Valve four was selected for measurement partly because of its intermediate position in the series of shell plates, and hence its possibly greater freedom from the operation of inherent growth tendencies of this character, but more particularly because, being the shortest of the anterior valves, slight erosional or other changes would produce greater percentage alterations in its length, which would thus be more easily detected. All that we wish to show here is, that the 'erosion point'—i.e., the average length at which the chiton individuals in a particular locality exhibit the effects of weathering upon the valves—can be determined and exhibited in a non-subjective manner. The irregularities in the relation between valve length and length of individual are due to the concomitant operation of these two tendencies, erosion at the beak and forward growth at the anterior mid-point of the valve, superimposed upon the normal growth of this structure.

Accompanying the erosion of the valves, which tends to produce upon their surface a chalky, brownish, or pale slaty hue, there occurs a plentiful accumulation of adventitious organisms upon the dorsal surface of Chiton (Plate, '01 a, pp. 380 ff.). The

<sup>5</sup> These matters are touched upon at this point for a reason which will become apparent further on in this paper. They are discussed with some completeness in a subsequent paper, by one of the present writers, dealing with the ethology of chiton.

kinds and number of these epizoites depend upon the size of the Chiton and the character of its habitat. The most conspicuous of them are barnacles (*Tetraclita*, fig. 1), *Spirorbis*, and *Serpula*. To this group must be added algae, comprising not merely the thin coating upon the valves, but also the *Enteromorphas*, which (in appropriate habitats) grow plentifully upon and between the

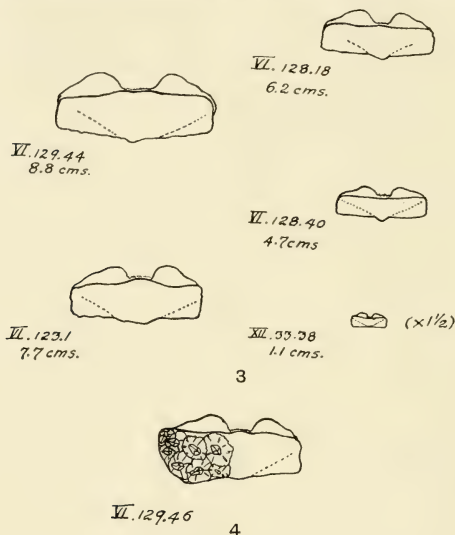


Fig. 3 Outlines of the fourth valves of five chitons of increasing ages (sizes); dorsal aspects, anterior up; see text.  $\times \frac{3}{2}$ .

Fig. 4 Illustrating protective growth of the valve substance resulting from the presence of epizoic barnacles; the outline is that of the valve.  $\times 1$ .

scales of the girdle. They are very important for the production of homochromic effects, because the periostracum of the scales is but little eroded, even in large chitons. The valves are rarely much overgrown with these algae, except among the largest chitons. In the felted matting of algae various young mollusks, nematodes, archiannelida, and protozoa abound. The serpulæ

affect only the very largest chitons. Barnacles remain attached to a valve in some cases until they have formed three growth lines ('year lines'). One effect of the barnacles is important in connection with our preceding remarks regarding the forward growth of the tegmentum as correlated with the erosion of the superimposed umbo. Instances such as that illustrated in figure 4 show how it is possible for the shell to grow in a protecting manner. In studying shell variation in the chitons it must be remembered that the attached barnacles may be removed, before or after their death, and leave no obvious trace, although they may have been responsible for irregular growth of a valve.

At sexual maturity the female *Chiton tuberculatus* is colored in a different way from the male: its tissues are impregnated with a salmon-pink substance concerned in the metabolism of the ovary. If the shell plates are separated, this differential coloration of the sexes may be detected in dorsal view. Normally, it is quite invisible. This is the first instance of its kind which seems to have been described among mollusks. Its importance has been discussed in another place (Crozier, '19).

In the gill channels and under the girdle of chitons obtained on sunlit shores where *Enteromorpha* and associated plants are growing in a felted covering over the rock, there are nearly always to be found considerable numbers of a commensal isopod. It appears to be the *Eusphaeroma* (*Sphaeroma*) *crenulatum* of Richardson ('02, p. 292; '05), described by her from specimens collected at Bermuda many years before by Goode, but concerning which no information as to habitat or local manner of occurrence has previously been recorded. The association of this isopod with *C. tuberculatus* is general throughout the Bermuda area, but the commensalism is of a more or less facultative kind, since the isopod is found sometimes among the algae at some distance from a chiton. Even where the supply of algae is scanty (as in crevices within the walls of caves), the isopods are also sometimes found, but usually not in such abundance, under the girdle of *Chiton*. As many as twenty or more are to be found under a chiton 8 cm. long. The association is quite independent of the sex and sexual coloration of the chiton. The isopods are



small (2 mm. long), and when taken from a chiton at low tide their coloration is quite pale, of a yellowish cast, with minute black markings. The coloration becomes darker in the light, and then reproduces on a small scale something of the greenish-to-black color pattern of the chiton girdle. The sphaeromas frequently remain in place under the chiton at high tide, and in a glass aquarium they will reassume a position within the gill channel or under the girdle of medium-sized or large chitons. There they take up stations chiefly along the lateral margins of the girdle, which are slightly raised during the respiration of the chiton. The isopods remain with heads pointed outward, into the incoming respiratory current. Sudden shading causes them to dart back into the ctenidial channel. When in their resting position a small portion of the anterior end may project beyond the edge of the girdle of their host, and under these circumstances their coloration, resembling that of the dorsal tubercles upon the girdle, renders them very inconspicuous. Occasionally one of these isopods creeps in between the gill filaments, usually at the posterior end of a chiton, and under these conditions is forcibly shot out at the posterior extremity of its host by means of the water current; the isopods appear, moreover, to react negatively to currents of this strength, and continue violently to swim away, in a spiral path, from the region of the anal current even after it has ceased to act upon them in a gross mechanical way. At high tide, and when under water in aquaria, the isopods creep freely over the dorsal surface of the chitons; under these circumstances their coloration is to a very high degree homochromic and concealing. This relation between *Sphaeroma* and *Chiton* will be made the subject of further study. At present it can be said that there does not appear to be any precise 'attraction' (chemical, for example) exerted by the chitons upon the isopods.

This commensal isopod is involved in the very complex environmental correlations, which may be clearly analyzed, in the life-history of Chitons. Therefore we mention it here, although detailed work on its behavior and relations must be deferred for the present.

*2. Growth and duration of life*

As with animals in general, the rate of growth of Chiton decreases with advancing age. For the area considered in this report the growth of *Chiton tuberculatus* appears to be adequately represented in figure 5. A detailed analysis of the material upon which this curve is founded will be given elsewhere.

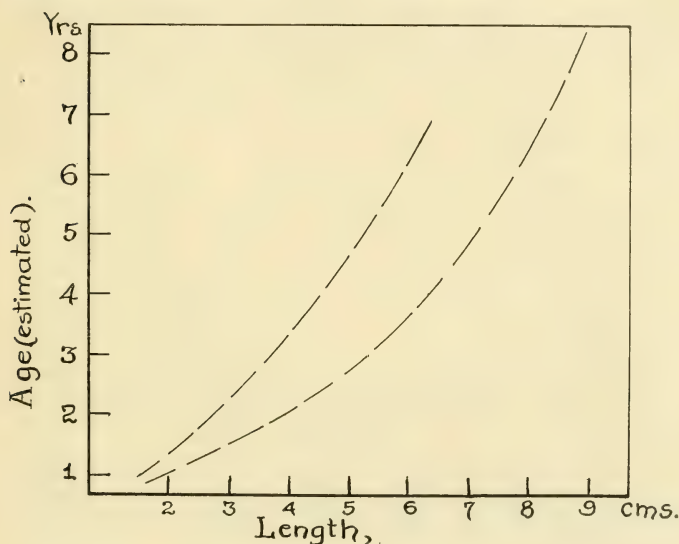


Fig. 5 Showing the relation between size (total length, in centimeters) and estimated age (years) in *Chiton tuberculatus*. These estimates are not intended to be of final significance. The normal average growth curve differs in shape from that shown. The two lines shown include between them most of the variations found in the *Chiton* population of Great Sound (April to May, 1918).

The maximal duration of life seems normally to be from eight to nine years (fig. 6). A length of existence so great as this appears not to have been suspected previously for the chitons. This species probably comes to reproductive maturity in the second (or third?) year of life (Crozier, '19). The general rate of growth

corresponds with that of some other chitons (Heath, '99, '05 b '05 c), at least for the early years. It is also true that in certain other chitons the second year marks the incidence of sexual maturity (Heath, '05 c). The large *Cryptochiton stelleri* grows more rapidly than *Chiton tuberculatus* does, but also matures in the second year (Heath, '05 c).

The curve in figure 5 is introduced here for the purpose of correlating some statements to be made subsequently regarding behavior at different ages (cf. Crozier, '18 b).

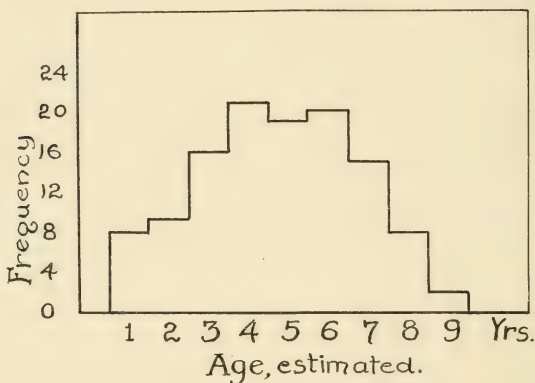


Fig. 6 The frequency distribution of estimated ages in the chiton population on the north shore of Long Island, Great Sound (April, 1918). It is necessary to consider each local population separately. The one here plotted is fairly representative.

### 3. Destructive agents

The power with which chitons may adhere to the rock surface is well known. When disturbed the girdle is firmly applied to the surface and the shell plates closely approximated. Except by means of a very powerful lateral push, it is impossible to dislodge them, once they are 'set' (which may happen very quickly); there is no projecting part of the smooth depressed animal which could offer a 'hold.' The dead plates found in the field have almost without exception been those of animals eight to nine years old.

Chitons are eaten as the main constituent of 'suck-rock soup' by some of the poorer people of Bermuda. One of us has observed rats quickly seizing chitons and devouring them. From the attacks of many other carnivorous animals of the shore zone, *C. tuberculatus* is relatively immune. About 7 in 1000 were found with oyster-drill holes in one or more valves. The animals whose shells were so attacked were always still alive. The holes pierced merely the tegmentum, the dense, hard articulamentum being impervious to the oyster-drill's efforts. Although as many as 125 barnacles have been noted upon one chiton of medium size, it does not appear that they produce a deleterious effect. After the death of the chiton, the barnacles drop off, frequently without leaving any trace; they are never very firmly attached, except in the case of very old chitons with thoroughly eroded valves, and it does not seem as though they can even pierce the periostracum. The same applies to *Spirorbis*. Serpulids grow only on very old chitons; they become incorporated in the substance of the shell, and appear to be in some instances responsible for a local increase in its thickness.

Injuries suffered by the girdle can be slowly repaired. Several animals were examined four weeks after they had been marked upon the girdle by having a deep notch cut in it. The notch had been partly filled in by new mantle tissue, the new dorsal surface bearing small, irregularly distributed plates. The new plates were at first widely separated and irregular in shape. After six weeks they were still irregular, but had become more closely set together. The power of regenerating the plates ('scales') may be related to the fact that the periostracum of these plates appears lustrous and uneroded long after the shell plates have been intensely weathered. Chitons are sometimes found in the field with small groups of the girdle scales removed, exposing the bare mantle, as well as with notches or 'bites' removed from the girdle.

The general impression derived from the consideration of destructive agents in relation to *Chiton* is that these mollusks are very efficiently protected. The length of life which they seem to attain, the variety of habitats which they frequent, and the

character of their sensory responses, which determine certain features of their life in these habitats, afford important evidence to this effect.

#### 4. *Feeding*

All the chitons, probably, are vegetable feeders. They rasp the thin coating of algae from the rocks by means of the radula (H. Jordan, '13), which is long, armed with powerful black teeth, and operated by a complex arrangement of muscles (Plate, '97). The body musculature is also involved in feeding. The whole body 'lurches' back and forth synchronously with the use of the radula, the forward swing coinciding with the retraction of the radula; the foot remains stationary. In rock crevices *C. tuberculatus* occurs frequently in groups, piled one animal upon another. Investigation has shown that under these circumstances they may feed on one another's backs upon the algae growing there. The radula removes not merely the algae, but some of the rock surface as well. The chitons may be of some slight geological importance in this way, and they may also be in small part responsible for the destruction of the periostraca of their associates and thus for the weathering of their shell plates.

Most of their feeding seems to be done at high tide. It is when covered with water that they move about most freely, although in damp places they also move to some extent at low tide. The great majority of the individuals are found well confined within tidal limits. While exposed to the air as the water falls they defecate copiously. The feces are discharged in the form of tiny cream-colored, cigar-shaped masses, varying in length with the length of the animal (fig. 1); in an animal 8 cm. long the fecal masses are 3.1 mm. long and 1 mm. in greatest diameter. The masses consist for the most part of minute granular bits of sand, but contain also undigested plant remains and fatty globules. When treated with acid, bubbles of  $\text{CO}_2$  appear; all but a slight meshwork of algae fragments is dissolved. The mass of plant fibers holds the fecal matter together in a pellet, which persists for as much as twelve hours under water in nature. When it is considered that, along the north shore of Long Island, for



example, more than 700 chitons, averaging 7 cm. length, were found within a strip three-eighths of a mile long, their eroding importance will be admitted to deserve examination. (A study of this matter is being made.)

By the time the tide has risen one-quarter, every chiton in an intertidal group is found to have deposited a considerable mass of fecal matter within the anal region of the mantle cavity. At high tide they do not appear to defecate to any great extent. There would seem, therefore, to be some rhythmic sequence of feeding operations roughly coördinated with tidal events. This might assist in the determination of a metabolic rhythm, which might in turn receive expression in (tidal) rhythms of behavior.

### 5. *Respiration*

The respiration of most individuals of *C. tuberculatus* is also subjected to the influences of tidal events. Under water, Chiton obtains oxygen by means of a water current, passing inward laterally along the girdle, through the gills, and escaping at the anal end (fig. 7). Out of water, the gills are more or less contracted against the dorsal wall of the ctenidial channel. Some oxygenation may, however, occur out of water, since the gills remain damp, and in nature the girdle is usually lifted from the substrate to some slight extent, unless the creature be disturbed.

The girdle is important for respiration, as the region in which it is lifted from the substrate localizes the intake for the water current. When completely submerged, this is commonly at the anterior end. The incoming water then impinges upon the dorsal surface of the proboscis ('palp'). Water is also taken in at the sides of the body. The latter is exclusively the case when the chiton is but partly submerged (i.e., with the anterior end out of water). The girdle may be locally lifted in the form of channels (fig. 7) or may be completely lifted. The water passes up between the gills, and escapes under an elevation of the girdle at the posterior end. This elevation is of somewhat variable form, although always located between the posterior ends of the right and left gill series. It is formed as a direct result of the water current impinging on the inner ventral margin of the girdle.

Figure 8 illustrates this point. When a chiton, partially out of water, on the wall of an aquarium, swings from a vertical position (fig. 7) to one such as that shown in figure 8, the posterior, elevated part of the girdle travels to one side as a smooth wave.

The water current also enables a chiton to sample the surrounding water. It is of importance for reproduction, since the stimulus to egg laying is provided by the diffusion of sperm from near-by males; these sperms are carried past the openings of the oviducts, past the 'osphradia' (p. 253), and eggs are liberated

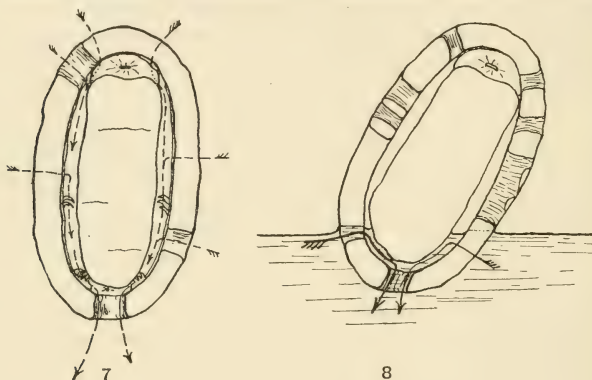


Fig. 7 Illustrating the course of the water current in Chiton. Diagrammatic.

Fig. 8 The course of the water current in Chiton when the animal is partially submerged. Diagrammatic.

in their company (Metcalf, '92; Heath, '99, '05 c). The nephridia also discharge their excretions into the respiratory current. These excretions, together with the water that has been 'used,' are usually shot to a considerable distance, because, the anal opening being smaller than the incurrent openings, the velocity of the outgoing current is high; here also, as in *Ascidia* (Hecht, '18), the 'used' water is discharged in such a way that it is not readily employed again for breathing purposes.

The ventral surface of the girdle is transversely ribbed, providing minute channels through which water is taken in, even

when the girdle is not detectably lifted; this can be demonstrated with suspended carmine. The girdle can, however, be very tightly applied to a smooth surface. A chiton, if attached to the wall of the dish, will live for two or three days completely submerged in an aquarium containing other dead and decaying chitons. During this time no water is taken into the gill channels. Hence, although chitons appear to frequent regions where, by wave action, the water is well aerated, it does not appear that they are particularly sensitive to want of oxygen.<sup>6</sup>

#### *6. Migrations; association in groups*

The larger chitons rarely engage in creeping movements unless they are at least partly under water. Occasionally they are seen to creep about when the wet under surfaces of rocks on which they may be situated are turned over and exposed to the light. They also creep, slightly, on wet rocks covered with algae. In dark pockets within the walls of caves, where compact groups of chitons may be found, they may be seen, if watched carefully, to move slightly upon one another; such places are, however, decidedly damp. When the tide comes in and covers a chiton, it may become active immediately. Conversely, when left by the receding of the tide, a chiton usually stops creeping and remains where it happens to be. If, however, water be splashed over it, it will continue creeping for a longer time; if the splashing be stopped, the animal stops creeping immediately.

Even when left in the sun to dry, upon the tide's falling, Chiton is not entirely immovable. In the case of the larger animals, if they be partially covered by a shadow, they will, even in this condition, move forward, or backward, or turn slightly, so as to become more evenly adjusted with reference to the light. The possibility of such movements suggested that an

<sup>6</sup> According to Heath ('05 c, p. 392), the gills of *Trachydermon raymondi*, which employs the gill cavities as breeding chambers, may become occluded during the breeding season by the 200 or more young trachydermons therein sheltered (Plate, '99, Taf. 6, fig. 218); under these circumstances the lateral proboscis lappets become (like the whole proboscis) much distended with blood, and may then be concerned in respiration.

'anticipatory' creeping toward the rising water of the incoming tide, based upon some form of hydrotropism, might be discovered in Chiton, and was accordingly looked for. None was found. Chiton is in this regard analogous to the actinians (Parker, '17 b); there is no 'memory' of recurring tidal events.

Having in mind the possible metabolic basis of tidal rhythms in behavior, discussed in connection with feeding and respiration, the behavior of Chiton has been studied for the occurrence of other tidal rhythms—inactivity as to creeping, movement out of water, and the like. Nothing of this kind seems to occur in *C. tuberculatus*.

Chiton, unlike a limpet, does not settle down into a depression closely conforming in outline to the impression of its shell. Neither does it, like a limpet, leave evidence upon the rock surface of wanderings and returnings to a 'home station' (Orton, '14). Inasmuch as a number of chitons seemed always to be present in certain depressions, or 'pockets,' which were examined at low tides, and since observation of the behavior of other chitons showed that they usually began to move about as soon as the rising tide had wetted them, data were sought to answer the question as to whether chiton exhibits in one form or another 'homing habits' of the type which have been described for *Patella* and its allies (Kafka, '14).

. An experiment of this sort is here recorded:

June 15, 1914. Observations were restricted to a definite area of smooth rocks below the boat house on Agar's Island. The chitons were marked for subsequent recognition by means of a deep notch cut in the girdle on either side of the body. (As noted elsewhere, about four weeks were required for such notches to be even partially obliterated through regeneration.) In the area of shore concerned in this record there were several deep crevices and niches into which chitons crept. The observations begun at this date were continued until July 14 (see table 1).

This table shows plainly that for a period of twenty-six days no material additions were made to the chiton population of this particular section of the shore, although it did appear that there were occasional new arrivals. In all, twenty-four chitons were marked, and at the end of the experiment, twenty-four days after

the last one had been marked, eight of these still inhabited the restricted region which was examined. The occasional arrival of a new chiton in this area is consistent with the gradual and fluctuating disappearance of the marked individuals. Perhaps the handling and stimulation due to cutting for marking purposes caused an initially increased wandering of the marked

TABLE 1  
*Concerning the migration and 'homing habits' of Chiton*

DATE	HOURL	TOTAL NUM- BER SEEN	PREVI- OUSLY MARK- ED	NEWLY MARK- ED	REMARKS
6/15	11.30	6	0	4	(1 lost; 1 injured and rejected) 10 found in deep crack; these overlooked before?
6/15	6.00	16	3	10	
6/16	10.00	12	11	1	A new niche found, overlooked before; both marked and unmarked animals were in it.
6/16	5.30	18	12	6	
6/17	11.00	10	9	1	One individual in a crevice, could not be seen well. A new one?
6/17	6.00	13	12	0	
6/18	11.30	14	13	1	Tide not completely down; hard to see clearly.
6/18	6.15	7	7	0	
6/19	9.30	9	9	0	
6/19	6.30	10	9	1	
6/20	10.00	7	7	0	One hidden; impossible to distinguish whether marked or not.
6/21	11.00	8	7	0	
6/29	11.00	12	9	0	Three others seen but inaccessible; marked?
7/ 1	6.00	10	8	0	Two others seen but inaccessible; marked?
7/14	6.00	9	8	0	

specimens. The general result is clear, however: Chiton is not stationary, it does move about to some extent, but adult animals, such as those used in this experiment, do not move frequently from place to place.

Further observations showed that there is probably some correlation with age in the matter of migration. The youngest



animals tend to occur in isolated groups, and further from high-water level than the younger individuals, there results a type of segregation which is favorable to the occurrence of some degree of homogamy (assortive fertilization). This would result in the economical utilization of sperms, and might possibly have additional effects of an 'adaptive' kind. Some further correlation between habitat and breeding habits in other chitons have been noted by Heath (05 c, 07).

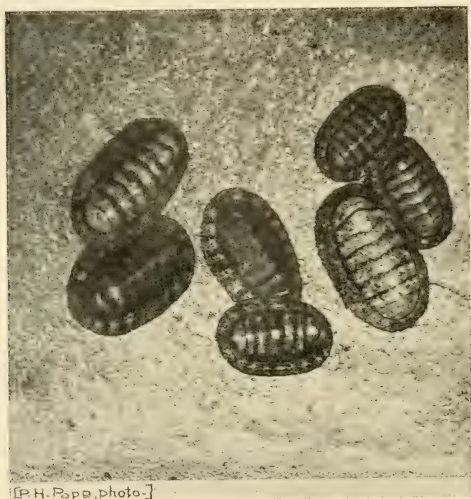


Fig. 9 A group of Chitons in a shallow depression.  $\times \frac{1}{3}$ .

#### 8. *Bionomic correlations*

*Chiton tuberculatus* is strictly intertidal in habitat. It, therefore, becomes possible to examine the details of its natural history rather extensively. The complexity of the catenary systems of relations revealed by such examination renders orderly description difficult. Some of these relations we have referred to in the preceding sections. Numerous others remain

to be considered. They concern phenomena of coloration, reproduction, determination of 'choice' of habitat, and similar features, comprising some of the things which involve explanation in terms of the animal's sensory physiology. In the matter of coloration, for example, the chitons in general exhibit homochromic ('concealing') characteristics which are commonly of some precision (e.g., in *Cryptochiton*, Heath, '05 b, p. 213, and in other genera which we have observed; cf. also Plate, 1901 a, p. 376). In *C. tuberculatus* this homochromic correlation is decidedly evident—in most cases it is unmistakable. It involves several pigments, their mode of distribution, the overgrowth of the shell by algae, barnacles, etc., a shifting of the chiton during growth to stations further below high-water level, the erosion of the valves, and a further shifting to more exposed habitats, with corresponding changes in the appearance of the creature. There is little reason to doubt that in the later growth of the chitons (three to four years old) conditions of food supply directly determine through the course of metabolism the character of the pigmentation displayed in the periostracum. The most fundamental factor concerned in the changing habits and appearance of chiton with advancing years, however, is its movement into more illuminated areas. The whole problem of its bionomic correlations becomes, from this standpoint, somewhat more directly open to attack. In general, it is not: How are the bodily processes kept going by the aid of movements? and, How does it happen that the movements are of such a character as to keep the processes going? (Jennings, '07, p. 57), but rather: What is the relation between the sensory capacities which determine and direct the bodily movements, on the one hand, and on the other hand the way in which the bodily processes are found actually to be going?

Naturalists have long been content to assign a given 'reaction' to some one or another of the categories of adaptation, and to rest satisfied that progress had thus been made in explaining it. No progress can be made in this way. Neither are we greatly helped by placing the responsibility for the adaptation in a general way upon the environment. The situations requiring

analysis are too specific. The real problem is to trace to their sources of origin some of the harmonious correlations—involving habits, coloration, and the like—which specific organisms display. For studies of this kind *Chiton tuberculatus* affords eminently advantageous material. The analysis of its sensory characteristics, forming the body of this report, is taken therefore as the starting point for a series of quantitative investigations in ethology.

### III. MOVEMENTS AND REACTIONS

For the analysis of the sensory capacities of *Chiton* we depend upon its motor reactions under various forms of activation. It is therefore necessary to outline the different modes of response exhibited by these animals. The movements of chitons present, in fact, a certain degree of diversity, somewhat at variance with the traditional epithet 'sluggish,' so frequently applied to them. Slow, as a rule, the movements undoubtedly are, and for that reason particularly favorable for examination, as the responses can be studied with precision. The motor reactions of *Chiton* comprise movements of local parts of the body, bendings and twistings of the animal as a whole, and pedal locomotion. This classification of movements is largely artificial, but convenient. Each of these classes may be dealt with in further detail.

#### *1. Local movements*

Local responses may be obtained from almost every part of *Chiton*. Since the muscular organs concerned in these movements are described in Plate's monograph ('97, '99, '01 a), they will not be considered here. The girdle (fig. 10) reacts locally by puckerings and by bending movements. The individual shell plates may be pushed apart from one another, elevated, depressed, and closely approximated. These local responses are involved also in the general movements of the whole animal.

The local movements of the ventral parts are less directly involved in responses of the animal as a whole. At the anterior end (fig. 11), the mouth, a transverse slit, is situated upon a

proboscis (fig. 14), clearly marked off from the foot. The periphery of the proboscis is thin and very mobile, and reacts by local contractions and bending movements when irritated. During feeding the mouth opens in rhythmic fashion to permit the extrusion of the subradular organ and lingual ribbon, as Heath ('03) observed in *Cryptochiton*. The whole proboscis may be retracted and temporarily covered by the forward extension of the anterior part of the foot; Heath ('99, p. 579; sep., p. 4) noted that the proboscis of *Ichnochiton magdallensis* was completely exposed in animals up to 4 mm. in length, but that with further growth it became normally covered by the pigmented anterior part of the foot. The surface of the foot itself is locally reactive, as shown by puckerings upon its surface and along its margins due to contraction. The substance of the foot may be swung, either as a whole or in any local part, to one side or the other, and may also be considerably extended as well as tightly contracted. The gills respond singly to local stimulation by contracting in such a way as to be pulled dorsally toward the wall of the gill channel. They may also, under certain circumstances, exhibit synchronous movements. The anal papilla is capable of movements of extension, retraction, and sidewise bending.

The neuromuscular mechanism of these movements is to a large extent locally contained. If the head segment or the tail segment be cut off, the tissues in the piece removed (head, sole of foot) are reactive to touch; stimulation of the foot or of the mantle causes the foot to be drawn toward the source of irritation. The part of the chiton remaining after the amputation also gives the customary responses, although the gill reactions are usually weak. The 'shock' effect of such an operation is of course severe, and is probably even greater when an animal is bisected transversely. In this case both halves are reactive, but the amplitude of the responses is much decreased, the gill responses to touch being absent. The local 'reflex' character of the regional movements is fully substantiated by experiments to be described on subsequent pages. This condition is reflected in the nervous architecture of *Chiton*, the central nerv-

ous apparatus being relatively unconcentrated and containing ganglion cells along the length of both pedal and pallial nerve strands. The large size of *C. tuberculatus* and the fact that it

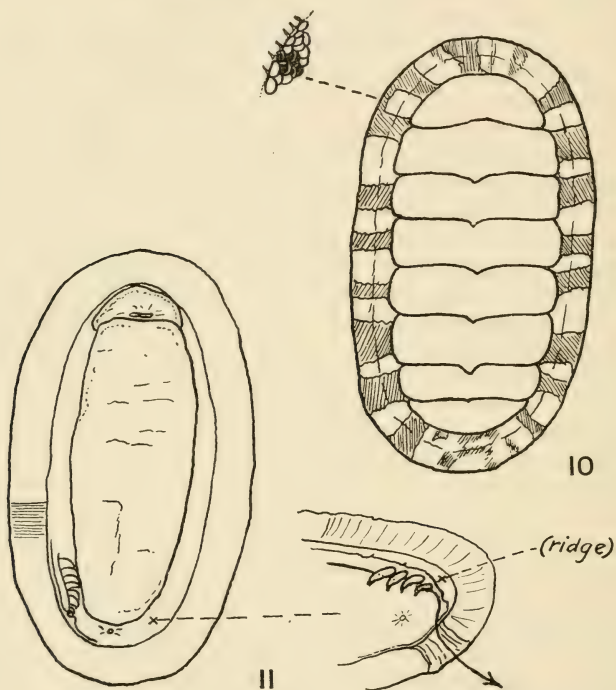


Fig. 10 Outline of a medium-sized *Chiton tuberculatus*, dorsal aspect. The detail drawing of a part of the girdle is magnified diameters.  $\times 1$ .

Fig. 11 The same, ventral aspect.  $\times 1$ .

will remain in an active condition out of water for a long time make it possible to study the local reactions in this species with considerable detail.



*2. Movements of the animal as a whole*

Probably the most striking general reactions of chiton are the suction process, whereby the animal adheres tightly to the rock, and the curling up, armadillo-fashion, which it exhibits when detached. Locomotion has less frequently excited remark (Cooke, '95, p. 400; Heath, '99, p. 579; sep., p. 4).

The suction power of chiton is well known to collectors (Dall, '07, p. 23). When the animal is disturbed, the girdle is applied to the substrate over its whole length, the shell plates are closely approximated, and suction is also exerted by the foot. The girdle is, however, the most important organ concerned in this protective response. Its efficiency is in part conditioned by its flexibility and by the fine riblets upon its ventral surface, but especially by the fact that it is morphologically differentiated into two concentric rings. This differentiation is exhibited in the coloration of the girdle, a narrow pale line being frequently located immediately inside the peripheral half of the girdle breadth. When firmly attached, a depression appears along this line, the more peripheral zone of the girdle being applied to the rock, and the inner zone being then sharply arched (figs. 10 and 11). On a smooth glass surface a chiton may readily be pushed about from side to side; in this case the foot is not exerting any suction, although the animal seems to be as firmly attached as ever.

The girdle is important not only for protection as a 'holdfast,' but also because it prevents the entrance of rain-water and of sand into the gill channels. Rain-water is quite toxic for Chiton, killing it in about four hours when the animal is placed upon its dorsal surface in a liter of such water. However, chitons will live for twelve hours or longer completely submerged in rain-water, provided the foot and girdle are free to come completely into contact with a solid surface; we have already noticed the completeness with which a foul solution can be excluded in this way. Chiton rarely frequents situations where it might be covered with sand, but occasionally it is left by the receding tide with the girdle and more or less of the back so covered. The

girdle is then closely applied to the substratum, and although, while under water, a respiratory current may be demonstrated, no sand grains gain admittance to the gills. The riblets and tiny channels normal to the girdle margin are important in this connection. If during creeping a lightly sanded spot is encountered, the girdle acts immediately as a plough, causing the sand to be pushed to one side. Although the tactile response of the girdle is thus very delicately adjusted, the commensal isopods (p. 167) are able to insinuate themselves beneath it without (usually) inducing any response.

The 'rolling up' of the body is the activity of chiton most frequently mentioned in descriptions. The animal when detached from the rock, even in the case of the smallest specimens, usually bends the head end sharply ventralward, the curvature of the posterior end following, so that the body becomes ultimately rolled together, the anterior edge of the girdle sometimes being beneath the posterior extremity, at other times the two ends being simply in close contact.

This response might conceivably be of significance in the life of chiton. When placed upon its dorsum on a smooth surface, it is impossible for *C. tuberculatus* to right itself. When rolled together, however, it could easily be moved by wave action to a location more favorable for righting. Moreover, the dorsal surface of the valves being sharply arched in the mid-line, the animal automatically rolls over to one side. This results in righting behavior somewhat similar to that evidenced by *Holothuria* (Crozier, '15 b). That it is ever actually resorted to in nature is quite improbable. It seems merely that the 'curling' is an unnatural result of the tendency to maintain the foot in contact with the substratum, its protective appearance and functional value in righting being illusory.

The flexibility of the body shown in 'curling up' is also evident in other movements. Although the plates are closely articulated, some sidewise bending is nevertheless possible. The animal may also become arched dorsally to a considerable extent, as well as bent sharply in the ventral direction, at any level. This flexibility is rarely shown in the natural habitat of chiton

except when it is creeping over a sharply curve surface. There seems to be a pronounced tendency for the avoidance of uneven tensions among the muscles. The normal place of residence is upon a flat surface; the somewhat unexpected flexibility of the body is nevertheless important, since it enables the girdle and foot to remain, during creeping, in close contact with the substratum, even though the latter be quite irregular.

The flexibility of the body is more evident in a form such as *Ischnochiton purpurascens*. This animal is long and narrow. It creeps with unexpected freedom, drops from one rock surface to another, when stimulated by light, and rights itself easily.

### 3. *Locomotion*

The locomotor activities of Chiton demand a few words at this point, and we are able to add slightly to previous descriptions of its pedal movements. *C. tuberculatus* characteristically progresses in an anterior direction. This is accomplished by means of pedal waves which are of a retrograde character, coursing from anterior to posterior as the chiton advances (Parker, '11, '14); in this respect it resembles another placophoran studied by Vlès ('07). As Parker observed, however, *C. tuberculatus* can also make backward movements of limited extent. Olmsted showed ('17 a) that in these backward movements of chiton the retrograde direction of the pedal wave is retained, as is also true in the *Fisurella* which Olmsted forced to creep posteriorly for a short distance; this we can confirm both for the pedal wave in chitons constrained to creep posteriorly, as in Olmsted's experiment, by having merely a small part of the posterior region of the foot attached to a substratum, and also for the occasional backward movements which occur when the whole foot is attached. Lateral waves, or at any rate one lateral wave-like movement at a time, are produced on the foot when the animal is intensely stimulated on one side (Parker, '14); in this case we find that the pedal wave courses from the unstimulated to the stimulated side (i.e., it is retrograde), but it not noticeably lifted from the substratum in wave form. Similar movements appear at the ante-

rior end of the foot during active creeping. The lateral wave produces an appreciable sideways shifting of the whole animal. Parker ('14) also noted that "by swinging the anterior portion of the foot to one side and the posterior portion to the other; the animal can rotate its body with the middle of its foot as a pivot." We have observed that in the case of these turning movements, which are frequently employed by chiton, the anterior end of the animal is the one primarily and principally concerned; the anterior end of the foot is, as a whole, pushed over to one side and diagonal retrograde waves bring the rest of the foot into the new position. The posterior end of the foot is pushed, as a whole, toward the side opposite the anterior one, but relatively not so far. During the turning maneuver the shell of the animal and the girdle are usually left behind, but after one or two pedal waves have passed, the foot (now straight) is held stationary, while the whole body of the chiton is swung slowly into the new position. We have spoken of diagonal waves upon the foot during turning; these waves are diagonal so far as the anterior end of the foot is concerned, but they usually become almost perfectly transverse before they reach the middle of the animal's length.

From the foregoing account it will be seen that there is, in comparison with most gastropods, a considerable degree of flexibility as to the use of the foot as a whole and as to the nature of the muscular coördinations producing pedal waves upon its surface, although this flexibility does not by any means involve such complexity of movements as appears in the foot of the gastropod *Cyprea* (Olmsted, '17 a). In the main *Chiton tuberculatus* progresses anteriorly by means of retrograde pedal waves; these waves in their characteristic form run almost entirely lengthwise on the foot from the anterior end backward and are not free to course in all directions across the foot as they are in the pedal disc of sea anemones (Parker, '17 b). Undoubtedly, this difference in the character of the pedal waves in the two cases is determined by the nature of the nervous arrangements within the pedal organ, and in fact the disposition of the nervous system of chiton allows us to analyze the relation experimentally.



During ordinary locomotion one or two waves appear upon the foot of Chiton; usually two waves are present when the animal is engaged in turning, but even in the absence of pivoting movements, one wave may appear at the anterior end before its predecessor has reached the posterior extremity of the foot. These waves are 5 to 7 mm. in anteroposterior extent, and involve the full breadth of the foot. They require fifteen to thirty seconds, usually about twenty-five seconds, to pass from one end of the foot to the other. The speed of progression of the pedal waves is less at lower temperature; it is identical in either sex, provided the chitons are of the same size. At 27°C, the speed of propagation of the wave is usually about 12–15 cm. per minute, being therefore faster than the rate of movement of the pedal wave in actinians (Parker, '17 b, 1 to 3 cm. per minute). The pedal wave is a region, occupying about one-tenth to two-tenths the area of the foot, which is temporarily lifted from the substratum (Olmsted, '17 a) and locally moved forward by muscular contraction. In backward locomotion, which may readily be induced by partial illumination of the shell, the retrograde character of the pedal wave is retained. This is especially evident in *Ischnochiton purpurascens*, which creeps freely backward if stimulated by horizontal light striking the anterior end of the shell.

In chiton there can sometimes be seen a distinct longitudinal depression running the full length of the foot in the midline, as if the foot were about to be folded together lengthwise. This is more easily seen in *Ischnochiton*. No trace of this activity is apparent in the pedal waves, however. Nevertheless, it can be shown that the foot is controlled in a bilateral manner. If an incision be made into the foot sufficiently deep to divide the connectives which join the pedal nerve strands, the lateral halves of the foot exhibit independent wave movements. If such an incision is made at the posterior end, a normal pedal wave may bifurcate when it reaches the anterior end of the incision, one half of it becoming obliterated while the other half may continue. 'Stationary waves,' sometimes opposite, sometimes unilateral, appear on a foot completely divided in this way; four or five such waves may be present at once, 6 to 7 mm. apart.



That the essential nervous mechanism of progression is locally contained within the foot is shown by the fact that when completely excised the foot will exhibit spontaneous wave motions; usually such a foot (which will live for two to three days in seawater) does not actually creep for more than a centimeter. The isolated foot reacts locally at its margin and on its ventral surface to touch, in the latter case giving well-defined suction responses. The pedal waves formed by the isolated foot are normal as to their speed of transmission; moreover, they appear one at a time, in succession, as in ordinary creeping; usually two or three waves exhaust the foot for half an hour.

The foot of placophorans, as of gastropods, serves also as a holdfast (Parker, '11), either by means of slimy secretions or through the action of the foot as a sucker. Parker ('14) pointed out that in *Chiton tuberculatus* the foot sucks locally, so that "if to the foot of an inverted chiton a rigid body with an area 5 mm. square is applied, the animal can attach itself to this area with sufficient strength to allow its weight to be lifted." As we shall point out subsequently in this paper, this 5 x 5 mm. area is about the minimal surface to which the *Chiton* foot will react by attachment and suction, so the full physical efficiency of its suction cannot, perhaps, be measured in this way. A chiton of 8 cm. length weighs approximately 50 grams, so in Parker's experiment just cited the foot was probably exerting a suction pressure of not more than 2 grams per sq. mm.—considerably less than the almost perfect suction efficiency of the tubercles upon the column of *Cribrina* (Parker, '17 e).

These observations indicate that, although the chiton foot is employed as a hold-fast, the foot itself is not sufficient to account for the full suction power of these animals. The tenacity with which they adhere to a rock surface is sufficiently remarkable to have gained for them the local name 'suck-rocks,' and in a preceding section we have shown how the girdle is of prime importance in this connection. An individual from which the girdle has been completely removed may with relative ease be separated from a stone over which it has been creeping. This is also true if a chiton is caused to become attached to a glass plate in

which there is a hole, provided the hole be situated beneath the gill channel.

During its normal existence, however, the foot is of course the organ whereby the chiton maintains its position. The whole girdle is often, especially when under water, completely removed from contact with the substratum; the support of the animal depends, in fact, almost entirely upon the foot. From the ease with which the chitons preserve their position in places where wave action is considerable, and upon the under surface of rocks, or (as has been noted through continuous observation) upon the relatively smooth vertical wall of a concrete wharf for periods of more than five months, it will be seen that the working power of the foot is, after all, adequate for the creature's needs. Chiton gets the most possible out of this suction power of its foot by keeping its whole area closely pressed against the substratum. Since it commonly inhabits smooth rock surfaces, the foot usually exhibits no great unevenness when the animals are freshly examined; but they are occasionally obtained creeping over bits of stone or groups of small *Modiolus* or barnacles, and if these individuals are inspected it is to be noted that the whole surface of the foot has been thrown into blebs and deep depressions corresponding closely to the unevenness of the substratum. Some of the blebs produced under these circumstances clearly demonstrate the basic mechanical principle upon which the foot works, for they appear as thin-walled vesicles filled with fluid (in the females, orange in color like the coelomic juice).

Similarly, if a Chiton be caused to creep over a small hole (4 to 5 mm. diameter) in a glass plate, the substance of the foot will be perceptibly pressed into the hole. Apparently the foot of chiton can exert suction only in a very local fashion, for if a portion of glass tubing of 8 mm. internal diameter, 10 mm. external diameter, corked at one end so as to provide a cylindrical chamber 4 mm. high, be used to test the sucking power of the foot, it is found that in most cases the chitons cannot become attached to the circular rim of the tube with sufficient force to bear their own weight in air. In this experiment, it should be noted, the total area available for direct contact with the foot is about 28

sq. mm., agreeing with the minimal area for attachment as found by Parker and in our own tests. The local character of the suction mechanism of the foot is further suggested by the minute depressions, usually long and narrow and more or less communicating with one another, which are to be found on the foot of a non-creeping Chiton attached for some hours to a glass plate in air. These local suction is probably assisted by slime secretion, which, although small in actual amount, enables a chiton to remain rather firmly attached to a smooth surface (e.g., of a glass plate) after the animal has been allowed to die in air or after it has been killed by heat (44°C.) in water. They do remain so fixed, even when the girdle is not in contact with the substratum, and the slime may therefore be important during the use of the foot in life, including early postlarval stages (Heath, '99, p. 640; separate, p. 65).

#### IV. MECHANICAL EXCITATION

##### 1. *Tactile stimulation*

In testing the local sensitivity of Chiton to tactile excitation, use was made of a blunt-pointed dissecting needle, a glass rod, or a blunt pencil-lead. In some instances, also, minute air bubbles (formed at the end of a pipette) and several other means of stimulation were employed. The responses observed when different regions of the dorsal and ventral surface of Chiton were lightly touched with one or the other of these objects are described in the following summary. Attention was given to the possibility that the responses of Chiton might vary depending on whether the animal was submerged in water when tested or was out of water. There were discovered no differences in behavior which require consideration at this point when the reactions of chitons in these two situations were compared. For the study of the responses obtainable from the ventral surface, we have mostly employed animals in air, placed upon their dorsal surface. The inability of chiton to right itself, coupled with the relative insensitivity of the shell surface, allowed us to work in this way without introducing serious complicating disturbances.

In the more critical experiments we made use of a method of graphic registration, subsequently described.

*A. Dorsal surface.* *a. The shell plates* appear not to be sensitive to touch. No responses were obtained when the surface of the tegmenta was lightly touched. (This is further considered on a subsequent page.)

*b. The mantle between the tegmenta*, i. e., the tissue covering the insertion plates, may be somewhat exposed, when Chiton is attached and 'at rest,' by the separation of the shell plates through the extension of the body. When the mantle was touched in this region the plates immediately adjacent to the site of stimulation were quickly approximated, covering the mantle area which had been touched.

*c. The girdle.* When Chiton is attached, the lateral extension of the mantle, known as the 'girdle,' which is flexible, is locally lifted from the substrate unless the animal be disturbed. Under water the girdle may be completely removed from contact with the rock or other surface, but in air this elevation is usually local and commonly takes the form of slight puckerings of, at most, a centimeter or so in length. To a single touch the girdle, where elevated, responds by local lowering to the substrate at the point of excitation. A more vigorous touch causes a greater extent of the elevated girdle to be lowered. Four or five moderate touches in succession affect a still greater length of the girdle, as much as one-quarter to one-third of the circumference, and the time elapsing before recovery to the original elevation is longer than that following a single touch. Even when the girdle has not perceptibly removed from contact with the substratum, it responds by a detectable 'tightening,' or flattening. Several successive touches upon a 'flattened' region of the girdle induce near-by elevated parts to return to the substratum. Unless the excitation is continued for nearly one minute, however, or is in the first place very vigorous, the response to touch is strictly homolateral. The anterior end of the girdle is more reactive than the middle or posterior parts, and its peripheral border is more sensitive than the rest of its dorsal surface.

A chiton quietly creeping in water, with the girdle lifted, re-



sponds instantly to a touch upon the anterior or posterior girdle by ceasing locomotion and adhering firmly to the substratum.

*B. Ventral parts. d. The ventral surface of the girdle.* The serial arrangement of eight dorsal shell plates affords a convenient means of dividing the surface of the animal into definitely delimited areas for reference. We shall consider the ventral surface of the girdle in terms of four 'quarters,'—an 'anterior quarter,' delimited by the posterior margin of the second shell plate, two 'middle quarters,' and a 'posterior quarter' correspondingly marked off by the transverse borders of each succeeding two shell plates. The end quarters of the ventral mantle are more reactive to tactile excitation than are the middle quarters.

*α. The end quarters.* To the single stimulation of an end quarter, the response is a curling of the animal in that region, as if it were beginning to roll up; the bending process elevates the stimulated end about 2 to 5 mm., after which the animal straightens out again. When stimulated on the posterior quarter, the foot may be pushed caudad at the time the bending response occurs. When stimulated on the anterior quarter, the head may retract somewhat, and the buccal region may be slightly inverted, the head and 'palp' tending to close over the mouth.

Light touches, when several times repeated, elicit a much stronger response. The stimulated end reacts first, by bending, and then the opposite end bends also, though to a less extent. This is true whether the anterior or the posterior end is the one stimulated. The muscles of the midportion of the animal are not specially contracted, however, and the closure of the shell is incomplete.

*β. The middle half.* The reactions listed under *α* are produced most clearly when the most anterior or the most posterior region of the ventral girdle surface is stimulated. The responses obtained from the 'middle quarters' of this surface are qualitatively identical over the whole anteroposterior extent of the ctenidia. In other words, the convenient descriptive division of the animal into 'quarters' does not afford a basis for the organic classification of responses, inasmuch as the ctenidia extend an-



teriorly and posteriorly beyond the limits of our 'middle half.' This artificial subdivision into quarters is retained in our description, however, since the responses we are considering are most characteristically displayed in the respective 'quarters' of the chiton's surface, although there is some 'overlapping' and, as already stated, the subdivision is by no means an organic one.

A single touch applied to the midventral surface of the girdle is followed by a local puckering of the girdle toward the source of irritation. The foot, in the region immediately adjacent to the level stimulated, is pushed laterad and dorsad, toward the mantle, tending thus to assist the girdle in covering the gills. This reaction of the foot is not evident when the dorsal surface of the girdle is lightly stimulated. Unless the tactile stimulation is severe or several times repeated the homolateral side only of the foot is involved in this response. Simultaneously with these movements of the girdle and foot, a contraction of the gill elements occurs opposite the singly stimulated area. This involves five or six ctenidia anterior, and as many more posterior, to the point of excitation. In this reaction the ctenidia are elevated dorsally, the tips are drawn toward their bases (thus throwing each element into a more convex arch), and at the same time they are drawn somewhat anteriorly. The whole response involves a movement something like the fairly rapid closure of the fingers of one hand. The response spreads in both directions from the level of stimulation, although at ordinary temperatures the propagation wave is difficult to observe because of its rapidity.

Successive stimulations of the girdle lead to a greater puckering in toward the source of excitation, and to a more pronounced rolling up of the whole body. The foot is locally brought slightly laterad toward the girdle and is drawn dorsad to a considerable extent. This response of the foot is at first confined to the stimulated side, but subsequently spreads to the other side, finally involving the whole substance of the foot at the level of stimulation. Successive touches, 1 to 1.5 seconds apart, lead to a tetanic contracture of the foot and ctenidia; during this phase the animal tends to roll up. A 'refractory period' succeeds the application of repeated light touches, until relaxa-

tion is fairly complete, during which touching locally does not evoke any response. The duration of this 'refractory period' depends upon the intensity of the original stimulation. The foot recovers very quickly and is not easily fatigued.

As in the stimulation of the dorsal surface of the girdle, the periphery of the ventral aspect is more sensitive than its more medial regions.

The most striking feature of these reactions is the strictly homolateral character of the response on the part of the ctenidia. In no case did the reaction spread to the gills of the unstimulated side. Homolaterality in response is not so clearly shown in the behavior of the foot.

*e. Tactile stimulation of the mantle lining of the shell* in the region of the ctenidia induces movements of the foot and gill filaments similar to those which follow touching the ventral surface of the girdle at a corresponding level. The responses obtained from the stimulation of the mantle lining of the anterior and posterior extremities are also similar to those obtained from the ventral surface of the girdle in the same regions, but the surface of the girdle is decidedly less sensitive. As with the girdle, no movements of the ctenidia are produced by tactile excitation of the mantle except along the anteroposterior extent of the ctenidia themselves.

*f. The region of the anus* is not excitable in any special way. To a light touch it may appear quite insensitive; to a stronger touch the anal papilla responds by local contraction on the side touched, and it may be somewhat retracted. When the mantle in this region is activated the behavior of the girdle and of the foot is essentially as already noted for other regions.

*g. The ctenidia*, when directly touched, respond as has already been described in the cases where the girdle and ventral mantle in the gill region were touched. Touching either dorsal or ventral surface of a filament results in the same response, although in one case it is directed toward, in the other case away from, the source of stimulation. Contraction in the manner previously described is the single mode of response of the gills. The base of each filament is more sensitive than the free tip. The

central 'rib' of each filament is by far the most sensitive portion; it is possible to pass a fine needle between the gill filaments without getting any appreciable response, although the filaments could be seen to move.

By employing a finely twisted bit of tissue-paper or a minute air bubble, it was possible to obtain the contraction of a single filament when delicately touched, although generally at least the two immediately adjacent ones were involved.

This result, taken together with the low tactile irritability of the lateral borders of the gill filament, shows that the antero-posterior spreading of the gill response when the mantle is touched is a nervous matter, and is not merely the result of one contracting filament mechanically involving its neighbors. This is also confirmed by tests upon animals having the pallial nerve strands sectioned at various levels. The operation produces no serious disturbance. The response of the homolateral gill series to fairly severe tactile irritation at one spot does not spread past the level of the cut 'nerve,' but ceases abruptly at this point (although the gills themselves have been quite undamaged).

The tactile sensitivity of the ctenidia is important for the efficiency of respiration. Foreign particles (e.g., sand) drawn into the gill channel by the respiratory current strike against the ventral surface of the gills, inducing a sudden local depression of the girdle, which squirts water out from under the girdle, removing the foreign object.

*h. The surface of the head*, the 'palp,' and the region of the mouth, are very sensitive to touch, the reactions produced being, however, merely local contractions; to more vigorous excitation, the animal responds by rolling up. The free margin of the head region is especially sensitive.

*i. The edge of the foot*, when touched in the region of the gills, induces responses such as those already described for the mantle lining of the ctenidial chamber. It is very sensitive. The posterior end of the foot yields responses like those of this region of the mantle.

*j. The sole of the foot* reacts by puckering away from a source of tactile irritation, such as a blunt glass needle. To larger areas it

responds positively by local attachment. The negative response of the foot to the touch of small surfaces is entirely local for a single stimulation. When repeatedly stimulated in the same spot, the primary local puckering spreads slowly across the surface of the foot, producing a dorsally arched depression 4 to 8 mm. broad, which is similar to the pedal locomotor wave, but generally deeper. Repeated stimulation also tends to produce the rolling-up response.

The anterior and posterior quarters of the foot are more reactive than the middle area. In these regions a single touch initiates a tendency to roll up, which can be produced on the mid portion of the foot only through repeated applications.

*C. Distribution of sensitivity.* The responses of Chiton when different parts of its surface are touched enable us to outline the distribution of tactile sensitivity over its body. In a general way the anterior and posterior ends—as is almost, if not quite, universal among animals—are more sensitive than the middle portions, and the peripheral parts than those more medially situated. Employing as criteria the relative effect in causing the animal to roll up, and, on the sides, the relative effect upon the gills, the following orders of sensitivity have been distinguished:

a. *The most anterior and the most posterior regions of the mantle are about equally sensitive.* It is difficult, if not impossible, to detect any constant difference in their reactivity to touch.

b. *At the head end: Surface of the head and palp = inner surface of mantle > ventral surface of girdle > dorsal surface of girdle.* The extreme outer margin of the girdle is about as sensitive as its ventral surface.

c. *At the caudal end: End of foot = inner surface of mantle > ventral surface of girdle > dorsal surface of girdle.*

d. *On the sides: Inside of mantle = ctenidia > edge of foot > ventral surface of girdle > sole of foot > dorsal surface of girdle > dorsal mantle between shell plates, the last judged by its effect upon the approximation of the shell plates.*

e. *The shell plates are insensitive to touch.*

It will be seen that in a broad sense the capacity of response to



tactile irritation is distributed upon the body of Chiton in a manner appropriately correlated with its structure and habits. The various responses obtained from contact with a small surface are such as would have a protective influence. Reference should be made at this point to the local closing together of the dorsal valves when the intertegmental mantle is irritated, and especially to the ventralward movement of the girdle, associated with local retraction of the ctenidia and a corresponding local movement of the foot, which follows a touch upon the dorsal or ventral surface of the girdle. The preservation of the ctenidia from injury, and more particularly the effective use of the girdle for the exclusion of foreign objects and as a hold-fast, are dependent upon responses such as we find the girdle to exhibit. Further detailed correlations of this character might be pointed out, but enough has been said to indicate the useful nature of the responses. The 'rolling-up' reaction has, when carried to completion, a clearly 'purposeful' aspect, as already intimated. Yet the natural history of Chiton yields no evidence that this response is ever used. We consider that it is the inevitable outcome of maximal possible contraction in the chiton's effort to produce suction, and that it is neither of specific protective significance nor of the nature of a 'reflex.' Confirmation of this view is found in the fact that sometimes a 'rolled-up' chiton will remain for hours tightly curled, although placed in position purposely made favorable for reattachment should it unroll. On the other hand, after a short time upon its back, a chiton may spontaneously uncoil itself and remain fully exposed for a long time, if unstimulated. Moreover, isolated parts of the animal give (or attempt to give, so far as their deficiencies permit) the 'rolling-up' response when they are activated.

*D. The tactile receptors.* The superficial layer of the shell plates of chitons is traversed by numerous canals, occupied by specialized organs having the histological appearance of sensory receptors. These canals are more or less nearly perpendicular to the surface (at least peripherally), and the organs they contain, piercing the tegmentum, are described as projecting slightly beyond its general surface. The remarkable character of these



structures is well known. Definite evidence as to their functional significance has been completely lacking. In addition to the 'eyes' (Moseley, '85; Plate, '99; Nowikoff, '09); micra- and megal aesthete organs of varied form are present, and some of them seem so constructed as to be (?) serviceable as tactile receptors. A function of this sort has in fact been somewhat doubtfully suggested for them (Kafka, '14, p. 100).

As already stated, however, the shell plates of adult chitons seemed insensitive to touch. A slight pressure, however, deforms the tissue underlying the plate, and is sufficient to induce a more or less pronounced sucking reaction. We tested therefore young *C. tuberculatus*, one to two years old, under the impression that in older individuals the aesthetes might be destroyed, as their cavities are exposed by the erosion of the cuticula. The result was again negative so far as tactile sensitivity of any part of the shell surface was concerned.

We then employed a method which completely avoided mechanical depression of the tissues beneath the shell plate. The free umbo ('beak') of the third or fourth valve was tightly gripped between the jaws of a haemostat. This did not involve damage to any of the soft parts. The forceps could then be clamped to an upright. By means of a small hole through the girdle or with the aid of cement attaching a thread to one of the anterior valves, the movements of the chiton could then be recorded graphically upon a kymograph paper. When the surface of a valve rigidly held in this way was explored with a needle or with a larger object, no tactile responses were elicited. (Shading must be avoided and rhythmic spontaneous contractions of the animal must be discounted.) Single touches and a moving point were alike without effect. There are no tactile receptors in the shell plates.

The histological nature of the sense organs in the tegmentum varies considerably in different genera of chitons (Plate, '99). Upon the shell of *Ischnochiton* there are minute, projecting 'hairs.' We find that the tegmentum of *Ischnochiton purpurascens* is very sensitive to touch.

These results allow us to state that in all probability the sen-

sory structures in the shell plates of Chiton have no functional significance as touch-receptors. The sensitivity of the girdle will be considered separately.

On the foot and other soft parts a moving point source of tactile irritation is very effective in inducing responses, its effect equaling that of several or many repeated single touches. On the soft surfaces, it would appear, there are many scattered tactile receptors. The amplitude of the reactions which they mediate depends, in any given region, upon the intensity of activation and upon the number of the receptors which may be involved.

The sense of touch exhibits also a certain degree of discrimination. Thus, to contact with small areas the foot reacts by local retraction, but to contact with larger areas it becomes promptly affixed. Furthermore, chitons in the field have been observed creeping in a horizontal direction along more or less vertical rock surfaces, just about at the level of the water at that time, so that wavelets of some force were hitting the animals roughly; they continued creeping quietly, the girdle being freely lifted, and made no response to the intermittent slaps of the water; but at the lightest touch possible with the finger upon the lateral or anterior edge of the girdle they instantly stopped moving and adhered firmly to the rock.

That the tactile sense is served by distinct receptors upon the soft ventral parts of Chiton may be shown through the physiological isolation of the tactile responses. Thus, when the ventral parts have been exhausted for photic excitation, by repeated shadings (*vide infra*), the mantle and other parts are still fully reactive to touch. When the ventral surface of the girdle was repeatedly touched, the animal responded by rolling up to the maximal extent obtainable with tactile stimulation; an additional vigorous response can still be obtained from the middle half of the girdle upon the application of  $n/10$  to  $n/40$  HCl, much greater contraction resulting from the use of acid in this way than can possibly be obtained through touch alone. When immersed in sea-water at  $43^{\circ}\text{C}$ . there was a considerable temporary augmentation of tactile responsiveness, but after a few minutes no responses to touch could be secured; a normal re-

sponse to a small volume of  $n/10$  HCl was, however, still obtainable. Taken in their entirety, these findings tend to indicate the physiological distinctness of the tactile receptors, although the methods employed in the tests are open to the objection common to all such experiments: we cannot be entirely sure that we are dealing with sources of stimulation which are quantitatively comparable. Less objection can be taken to the result of experiments of the following kind. If the inner ventral girdle surface, under water, be repeatedly stimulated with small volumes of  $n/30$  HCl in sea-water, four or five successive responses may be obtained from the activation of one area. This area is then exhausted for stimulation in this way. It is, however, still reactive to touch. Since the acid is usually regarded as a more powerful excitant than touch, the objection above referred to may thus be removed.

## 2. *Vibratory stimuli*

The characteristic statocyst of gastropods is not present among amphineurans. Hence it would be interesting to learn—although the theory of the statocyst as a merely positional organ seems now well established (Baunacke, '14)—whether or not *Chiton* reacts to vibratory stimuli such as sound waves. It proves not to respond to attempted stimulations of this kind.

Chitons placed in a beaker of thin glass containing sea-water were watched while the lip of the beaker was tapped with a glass rod. No reactions followed this treatment. Sounds transmitted to the beaker from a vibrating saw blade had likewise no effect. The table top supporting the beaker was sharply struck, jarred, or rubbed, with the same absence of response. Chitons placed ventral surface upward in shallow water, so that they were just covered, did not react to drops of sea-water falling on them from a distance of 10 to 15 cm.

A chiton resting upon a glass surface, under water, in some cases responded to a tapping of the glass immediately under the appressed mantle by raising the girdle in that region. If the girdle was already raised, in a few cases only was it lowered to the

glass surface as a result of the tapping. In several instances chitons treated in this way began to creep, and in one case began to creep backward.

There is no suggestion of the perception of vibratory stimuli in these results. Direct contact excitation is quite effective, but vibratory disturbances, such as sound waves, seem not to be reacted to. In collecting chitons it is advantageous to employ a large cold-chisel and a hammer; yet one finds that the removal of several members of a group, involving repeated and fairly heavy blows, does not usually result in near-by chitons becoming firmly attached to the rock, unless they have been directly affected by a deforming pressure. This deficiency cannot be attributed to the absence of a statocyst, however, since other mollusks, well provided with statocysts, are known not to react to vibratory disturbances.

A chiton may be suspended in water by having the beak of one shell plate clamped in forceps attached to a support. Under these circumstances it does not react to vibratory disturbances transmitted through the water.

### 3. *Thigmotaxis*

The surface of the foot of Chiton, if touched locally, draws away in a rather sharp pucker, especially if a sharp point be used. No attempt is made to attach the stimulated spot by suction, and if the activation is repeated the resulting movements of the foot are such as to cause its removal from the region of stimulation. If, on the other hand, a larger surface, such as the flat end of a pencil, be applied, the foot becomes firmly affixed to the foreign surface, and is pulled deeply down below the general level of the rest of the foot. The minimal area reacted to by attachment is about 5 x 5 mm. for chitons 6 to 9 cm long, as Parker found ('14).

It is important to note that when a pedal wave has formed and is traveling down the foot of a chiton lying on its back, the surface of the foot immediately involved in the wave can attach to a small point (e.g., the pointed end of a pencil) very firmly by suction.

When placed upon a glass plate the animal quickly puts its whole foot in contact with it. In doing this, waves are set up frequently and three or four may appear upon its surface at one time; or, if the foot is already in nearly one plane, the attachment may be almost simultaneous over its whole extent. In the latter case, the local areas of the sole which are not at first attached are brought down to the substratum.

As noted by Olmsted ('17 a), a chiton repeatedly forced to creep backward, by requiring one-fourth of the foot to attach to the lower edge of a glass plate held vertically in air, becomes after several trials exhausted, so that it creeps just sufficiently to enable the whole foot to be in contact with the plate.

Thus, to contact with a small area, such as a needle point or the rounded point of a pencil, the resting foot of Chiton reacts negatively, but to larger surfaces the response is a positive one. There is a very pronounced tendency to keep the whole of the foot in contact with some foreign surface. In no case has a chiton ever been seen in nature with any section of the foot or head completely removed from the substratum. This response is sufficient explanation of the behavior of Chiton in 'righting' itself. At no age is there a detectable tendency for chitons to preserve an upward orientation of their dorsal aspect, even when placed so that it is physically possible for the animal to reattach itself (*vide supra*, p. 200); undirected movements finally result in a portion of the foot, usually the anterior end, effecting contact with the substratum; complete reattachment is then rapidly brought about.

It is of interest to inquire if this form of tactile discrimination, favoring attachment to a sufficiently large area of surface, is evidenced by parts other than the foot. The ventral surface of the girdle and the head region were therefore examined.

Finer degrees of tactile discrimination seem to be absent on the head and foot. Chiton shows no preference, when placed in an aquarium, for surfaces such as those to which it has been accustomed. Provided the surface be firm and sufficiently large, it creeps indiscriminately over smooth stones sparsely sprinkled with sand, glass, or wet paper. It will not creep, however,



upon sand, upon mud, or upon a rock surface minutely studded with sharp points; the negative reaction to surfaces of this character is effective in determining the local habitat of Chiton, since they do not occur upon muddy beaches nor upon sand, nor do they at any time creep up upon the sharply pitted shore rocks in the narrow zone which is covered at spring tides but otherwise exposed to wind erosion. It may be noted in addition that the young chitons, up to three years of age or more, and especially in very young stages, occur conspicuously upon smooth stones. This is not altogether an accidental consequence of the fact that the rock surfaces in the situations where their tropisms force them to reside are frequently of a smooth character, since the smallest specimens are found upon the under side of bottles (of dark glass) below mean-tide level in company with *Ischno-chiton*.

#### 4. *Rheotropism*

It was noted that a number of chitons escaped from a collecting pail, located at one end of a long aquarium table, and that they tended to accumulate in the shallow gutter which carried away the overflowing sea-water. A good number of these animals moved down the gutter, with the current, even though in so doing they traveled slightly down hill, against their negative geotropism. Further tests, made in this gutter, showed that, to currents of sufficient strength to produce any effect, a majority of the animals were negatively rheotropic. For a more refined test, chitons were completely submerged in a trough of sea-water (a wooden fish-hatchery trough) through which a current flowing at the rate of 5 to 10 cm. per second was maintained. The rheotropic response was here less definite than it appeared in the first observations, but was undoubtedly negative.

The rheotropism of Chiton is clearly a 'laboratory phenomenon,' and may owe its appearance to the mechanical deformation of the girdle by currents or to other tactile irritations, in either case inducing negative orientation, or it may be traceable to a deforming influence of the current upon the body as a whole,

especially upon the base of the foot, such as we suggest in the case of geotropism.

The effects of local currents were also tested. A current from a pipette directed under the girdle of a submerged chiton induces a depression of the girdle, provided the gills are disturbed. A current directed upoh the girdle causes it to be depressed; it can usually be seen, in this case, that the 'scales' upon the girdle are moved or that the girdle itself is mechanically bent. A current impinging upon the dorsal mantle between the shell plates produces usually no effect. The girdle is the most sensitive region.

Negative reactions of the whole animal are readily induced by repeated applications of a pipette current to a part of the girdle. These reactions are also concerned, probably, in the orientation of Chiton in a vigorous stream of sea-water.

### *5. Geotropism*

Since the Amphineura lack the statocyst organs characteristically developed in other molluscan classes, the question of Chiton's behavior with respect to the pull of gravity deserves special consideration. The positions in which they are commonly to be observed strongly suggest that they are negatively geotropic. The younger individuals, particularly, are found most abundantly at the upper limit of the tidal reach. Older animals occur over the whole intertidal zone, and even in some cases slightly below it, but these, too, are most frequently encountered near the upper tidal limit. Furthermore it is very noticeable, more particularly among the larger individuals, that the great majority of the chitons taken from perpendicular rock faces are oriented with the anterior end upward, rarely indeed with this end directed downward, although in many cases they are more or less nearly horizontal. When kept in aquaria they rapidly creep to the water surface and sometimes thence out into the air; nevertheless, they sometimes curl over the top of an aquarium and creep downward on its outer wall (more especially in the case of aquaria with a free general overflow at the

top). During downward creeping their orientation is rarely such that the long axis is strictly vertical.

The following specific experiments show that the orientation of the movement of Chiton is generally in an upward direction.

1. Five chitons were placed, with their long axes horizontal, on the walls of a flat-sided aquarium jar. They were located at about midway between the bottom and the water line. The jar was removed to a situation where the illumination was even and diffuse. Three individuals moved upward to the surface of the water, and nearly emerged; one oriented upward through an angle of  $45^\circ$ ; the other one oriented downward through an angle of  $60^\circ$ .

2. Six chitons, placed as in the preceding test, were kept in the dark-room for 1.5 hours. At the end of this period four were found to have become oriented vertically, moving upward until almost entirely out of water, one was oriented upward at an angle of  $45^\circ$ , and one had moved upward to the water surface, being oriented upward at an angle of about  $45^\circ$  from the horizontal. Another similar test was made with eight animals, six of which oriented upward at various angles and two downward; in this experiment the animals were overcrowded and became piled upon one another.

3. A similar test with five chitons, kept overnight in the dark-room, gave four animals orienting upward and moving nearly out of water; the remaining one had moved downward, assuming an orientation  $45^\circ$  away from the horizontal.

Of these twenty-four individual responses, twenty were clearly such as involved an upward orientation from the horizontal position. Since the animals continued creeping until almost entirely out of water, it is hardly probable that want of oxygen determined the observed behavior. This conclusion is confirmed by subsequent tests in which chitons were placed in dishes where anaerobic putrefactive processes had begun or were allowed subsequently to begin. Frequently they did not creep out of such dishes. Moreover, Chiton orients upward in a closed vessel completely filled with water or one in which oxygenated water enters from beneath. The negative orientation is evidenced by animals of all ages. It is most perfectly expressed when the chiton is completely submerged. The horizontal position assumed at the water level is a special consequence of stimuli associated with the water level.

The tendency to upward creeping is clearly evident upon

surfaces inclined at any angle above  $30^\circ$  with the horizontal. For these tests the larger animals are best.

Observations in the field and many tests in the laboratory show clearly that there is no tendency for Chiton to preserve a constant dorsoventral orientation. In rock crevices they occur 'upside down' with great frequency.

Careful observation of the movements of a chiton during geotropic orientation affords a clue as to the nature of the determining stimulus. Accurate outlines of a specimen orienting in this way are given in figure 12. Inspection of these outlines will show that the sequence of events in orientation is as follows:

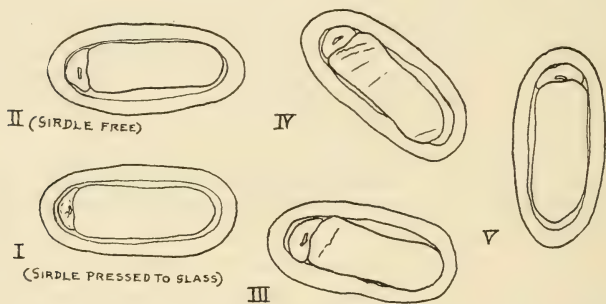


Fig. 12 Outlines of the successive positions assumed by a Chiton in orienting upward from a horizontal position, under water, on a vertical surface; ventral aspect. (Traced through glass, on thin paper.)  $\times \frac{2}{3}$ .

The girdle becomes freed from the substratum, so that the animal remains attached by the foot only; when in the horizontal position (fig. 12, II), the weight of the body causes it to fall slightly, producing an uneven tension in the muscles, those on the higher side being stretched. The animal swings until this unilateral tension is relieved. It turns anterior end up, probably because that end is the more sensitive. The tendency is for the animal to turn toward the stretched side; the tensor muscles are the ones which contract. With animals in the vertical position (fig. 12, V) the downward pull of the creature's weight is exerted at the posterior end.

The chief ethological importance of Chiton's negative geotropism is not that it directs the animal upward, but that it keeps it from going down.

The pallial nerve cords are easily cut by an incision from the ventral side. A chiton with both pallial nerve strands cut at the level of the cleft between proboscis and foot no longer orients upward; it creeps about slowly and aimlessly, almost invariably moving downward, but in a slanting direction. Sometimes a chiton so prepared turns upward, but in no case did one of the six animals used move upward, as normal individuals consistently do. When the pallial cord is cut on one side only, and the animal allowed to attach itself (after a rest) to a vertical glass plate, under water, the long axis of the chiton being horizontal, the characteristic effects are as follows: if the side on which the pallial nerve cord is cut is placed downward, the animal orients downward; if the cut side is uppermost, the chiton orients upward, but usually passes beyond 'top center,' and then frequently reverses, coming back to an approximation of its original position, but not quite so nearly horizontal. This reaction is not always clear cut. The final position, in fifteen tests upon twelve different animals, was in all but three cases such that the cut side was downward. In a number of cases there seemed a well-defined failure of the anterior portion of the foot to attach itself to the substratum, so that, the posterior region being fully attached, the anterior end became directed downward in a purely mechanical way owing to the pull of gravity. This might account for the fact that in the chiton with both pallial cords cut the anterior end is in a general way directed downward, but would not explain the fact that in most cases with the cut side placed upward the orientation was upward. While not conclusive, the result of these tests is for the most part in harmony with the idea that the weight of the body, exerting tension on the pedal musculature, supplies the stimulus to geotropic contraction. In the case of an animal placed on a vertical surface with the long axis horizontal, the muscles on the stretched side are the ones which contract, as we might predict, and since the animal orients upward, we must suppose this to be in a gen-



eral way true. If the nervous connection of one ctenidial wall of the foot mass by means of the pallial cord be severed, then the muscles on that side, passively stretched, or compressed, could not (or might not be able to) get readily into nervous communication with the rest of the body; hence we should under these conditions expect geotropic movements to be imperfect. No tests have yet been made to discover the effect of uni- or of bilateral section of the pallial nerve cord on horizontal creeping.

### *6. Summary*

The general surface of Chiton, aside from the tegmental surfaces, is actively responsive to touch. The aesthetes upon the shell valves are not sensitive to tactile stimulation. The foot is negatively reactive to small surfaces, positively thigmotactic to large surfaces. Chiton is negatively geotropic, and also negatively rheotropic to currents of some strength; these two modes of response appear to depend respectively upon the development of unequal tensions in the musculature, owing to the weight of the body, and upon the local deforming influence of a water stream—they result from stimulation through deforming pressure.

Although much has been written upon the morphological character of the sensory organs in Chiton and upon the general structure of the integument (Blumrich, '91; Plate '87-01 a), there has been very little in the way of experimental evidence bearing upon functional relations. Heath ('03) thought that the proboscis of *Cryptochiton stelleri* might exercise tactile as well as gustatory functions, and described some feeding experiments in support of this idea. From the foregoing account it will be seen that tactile (contact) irritability is a general integumentary function, leading to responses of an 'advantageous' character. The receptors concerned are apparently of several kinds: 1) those upon such surfaces as the foot, mouth area, and gills, and, 2) those associated with the tubercles and 'hairs' upon the girdle. The former are probably of a more generalized character than the latter, but, for some parts at least, the propo-

sition is nevertheless defensible that they are *tactile* receptors and that the responses to which they lead are not aspects merely of the activity of generalized ('universal') sense organs. The positive response of the foot to contact with a sufficiently large area is significant upon this point, since this type of reaction is never obtained upon chemical stimulation of the foot.

The tubercles and 'hairs' upon the girdle of most chitons are characteristically embedded in the cuticula of the integument and connected at their proximal ends with secretory and probably also with sensory cells. By means of movements of these stiff projections, relatively slight mechanical disturbances may be transmitted through the thick cuticula. We have shown that on the shell surfaces of *Ischnochiton* the projecting 'hairs' are open to tactile activation. In *Acanthochites spiculosus* the girdle is regularly beset with circularly arranged groups of long spicules. We find these spicules to exhibit an interesting reaction. If one of them be touched, ever so lightly, the rest of the spicules in that group react also, the whole bundle being spread widely apart. Usually all the bundles on one side respond if a single spicule is disturbed. As the spicules are 5 mm. long, in an animal 15 mm. long, a slight touch exerts a considerable leverage upon the base of the spicule. At rest the spicules are directed posteriorly, but when disturbed the axis of each bundle is directed more or less perpendicularly to the surface of the girdle, the spicules themselves being spread wide apart; this results in a disposition of their sharp points which may have protective value.

The 'scales' on the girdle of *Chiton tuberculatus* are homologous to the spicules of *Acanthochites* (Plate, '01 a), and their functional significance for tactile reception is of a similar kind. At the outer margin of the girdle are short, stiff 'hairs,' directed normally to the periphery, which also function in this way<sup>7</sup> (Plate '01 a, p. 497, believed the 'hairs' and 'thorns' of the chiton girdle to be sensory organs).

<sup>7</sup> Heath ('99, p. 637; sep., p. 62) has described the use of the anterior flagella of the transforming larva as tactile organs.

Chiton exhibits mouth movements similar to those figured by Heath ('03) for *Cryptochiton*. It is questionable whether these movements originate normally from tactile excitations. On a glass surface they are never observed except in starved individuals. The mouth opens widely, permitting the subradular organ to be pushed forward. In a starved animal these movements, which occur in rhythmic series, may be initiated by causing a bubble of air to become entrapped in the depression surrounded by the lips. The radula is never thrust forward sufficiently to come clearly into view. Full feeding movements are obtained, however, when a splinter of the intertidal rock surface is placed in contact with the proboscis. It is of course possible, as Heath suggests, that the rich supply of nerve endings on the surface of the subradular organ is in part of tactile significance.

#### V. THERMAL EXCITATION

##### *1. Behavior at different temperatures*

It is worthy of remark that very few marine invertebrates, if indeed any, seem to possess a well-developed temperature sense. Although as yet we know little of the integumentary senses in marine annelids, crustacea, or mollusks (Kafka, '14), we may note that in echinoderms (Crozier, '15 b; Olmsted, '17 b) sensory discrimination for heat and cold is very weak, and that even in *Amphioxus*, where there are good indications of possibly both heat and cold receptors (Parker, '08, p. 430), the responses of the animal to either heat or cold are by no means of that delicately sensitive character which we usually associate with photic, tactile, or chemical receptors. We believe this to indicate a poorly developed sensory mechanism for heat and cold reception. There is little reason to regard this condition as one of adaptation to, or correlation with, life in a situation where thermal receptivity would not be valuable, owing to the small range of temperature occurring in the habitat of the creatures in question. It would be equally sensible to consider that, if the animals concerned had developed deli-

cate thermo-receptors, some 'use' would have been found for them. In either event the adaptationist rests intellectually satisfied. It is, nevertheless, a fact, although it may have little bearing upon this matter, that the temperature variations endured by Chiton are rather wide. These variations are both diurnal and tidal, and their amplitude, in summer months, extends during the twenty-four hours in some situations from 23° to 37°C.; this is especially the case on hard bare limestone rocks, where the heat of the midday sun at low tide is quite intense. At the time these experiments were made the surface temperature of the sea was 26° to 27°C., and this temperature was taken as the 'normal' in the tests that are here described.

Chitons were immersed in sea-water maintained at the temperature noted, and their movements were carefully recorded.

*Behavior of chitons transferred from sea-water at 25° to 26°C. to sea-water at the temperatures indicated at the margin*

TEMPERATURE IN DEGREES CENTIGRADE	
2°	Ctenidia almost instantly contracted, and remained so for 3 min.; 5 min. from time of immersion, all were again expanded. Ctenidia respond to tactile excitation by contraction, but weakly. No tactile responses obtainable from other regions. No spontaneous movements; remaining as if anesthetized.
4°	Ctenidia contracted; began to expand in 2 min. At first, the foot partly curled, as if preparing to roll up, but soon became straight again. Tactile sensitivity soon abolished; after this had been ascertained the foot and mantle were tested with weak HNO <sub>3</sub> solution, but no reaction resulted.
5°	Ctenidia contracted within 3.5 min.; after 2 to 4 min. began to expand; fully extended after 4 to 5 min. Ctenidia respond to touch, feebly in some cases. No spontaneous movements of the foot or animal. Responses of the body to touch absent after 15 min.
8° to 10°	Foot spontaneously thrown into smooth contractions that lasted 2 min. Ctenidia contracted; after 4 min. they relaxed one by one. After 10 min. ctenidia responded to touch. General tactile responses very poor.
10°	Ctenidia contracted after 0.5 to 6.0 min.; expanded after 7 to 10 min.; after 10 to 11 min. reactions to touch returned, but very slowly. In several cases after 1 min. immersion the gills began

TEMPERATURE IN DEGREES CENTIGRADE	
	to contract rhythmically at intervals of 5 to 8 sec., continuing in this way for 2.5 min.; the gills of the two sides did not beat synchronously, but the plumes of either side alone were at first more or less coördinated, the unison of the 'beats' becoming less after 2 min. Foot exhibited few or no spontaneous contractions, but was much extended, exposing the ctenidia. The animals as a whole insensitive to touch.
15°	No contractions of the ctenidia; a few smooth contractions of the foot and slight movements of the palps for several min. Animals normally extended. Tactile responses subnormal.
20° to 35°	All responses normal.
38°	General sensitivity ('reactivity') slightly increased during the first few minutes. No spontaneous movements of the foot or palp. Sensitivity to touch rather quickly decreased, but still present (feeble on the ctenidia) after 45 min. Animal could not close the shell when stimulated (note that at higher temperatures the shell upon immersion shows a tendency to open, if it had been closed, i.e., rolled up).
40°	If shell is rolled up, it opens. Spontaneous writhings of the foot either absent or lasting 3 to 5 min. Ctenidia in some cases contract for 1 min., irregularly. For 1 to 2 min., ctenidia and other parts are extremely reactive to touch. Tactile responses gradually decrease; at first, a single stimulation of the foot (after spontaneous movements have ceased) induces several irregular contractions; after 30 min., foot alone is slightly responsive to single or repeated touches; but irregular contractions producing a welt result from moving a pointed rod over the surface of the foot. After 1 to 1.5 hour, still in same condition.
42°	Violent contractions of the foot for 1.5 min.; then ceased. After 3.5 min., all contractions ceased, and no tactile responses were obtainable.
43°	If shell was rolled up when immersed, it opened; few, and no successful, attempts to close the shell. Foot thrown into irregular contractions, originating as local puckerings, which spread rapidly, lasting for 2 to 2.5 min. These contractions not so convulsive as at 42°.
	After 1 to 5.5 min. (in one case following tests for tactile sensitivity) a second series of weak contractions of the foot appeared and lasted for 2 min. The shell plates moved slightly back and forth.
	In one instance there were a few irregular but widespread contractions of the ctenidia, in which each filament appeared to act more or less independently.



TEMPERATURE IN DEGREES CENTIGRADE	
	No reactions to touch obtainable after 2.5 to 4 min., from either foot, palp, or ctenidia.
44°	After 15 min., the foot and mantle are much bloated. No movements of the foot; no responses to stimulation. Chiton attached to a glass plate raised the mantle, except at either end, but remained passively attached, after death, for 10 min. Died in 2 min.
45°	Chitons straightened out and the shell and foot became convex. No responses. Died at once. Animals attached to a glass plate showed slight writhings of the foot when first immersed, due probably to slow warming up through the glass.

These tests show that sudden changes in temperature between 15° and 40°C. have little in the way of direct sensory effect upon Chiton when the whole animal, having previously been maintained at about 27°, is suddenly immersed in sea-water of any temperature between these limits. Below 15° an 'anaesthetized' condition is quickly arrived at (Matisse, '10); above 40°, sensitivity quickly decreases. Temperatures of 44° to 45° are almost instantly fatal, although Chiton will survive for nearly two hours after sudden transference to a temperature of 40°. This is the highest temperature which they will successfully withstand for more than fifteen to twenty minutes, and is but a few degrees above the summer temperature sometimes experienced for a similar period in their natural habitat. The factor of safety is here, consequently, very small, as compared even with that of other littoral animals of the tropics, which as a group live in the upper zone of temperatures compatible with life (Mayer, '14). The smallness of this safety factor, and the actual magnitude of the temperature quickly producing death, as compared with that for other forms frequenting near-by localities, is sufficient to show that there is little or no trace of adaptational modifications correlated with external thermal conditions. Thus, among animals which have been studied at Bermuda we find such facts as those set forth in table 2, where it will be seen that although there is an undoubted general correspondence in the upper temperature limits, or thermal death points, the correlation of these values with the

normal life conditions of the several animals involved is by no means precise. The temperature producing rapid death in *Chiton* is a little higher than that found for many shallow-water species in tropical seas (Mayer, '14), but the maximal temperature successfully withstood for a short time ( $40^{\circ}$ ) is not noticeably greater than that for other animals which do not live upon sun-baked rocks. Shelford ('16) has insisted upon the correlation between the survival time of organisms (of the same and of different species) at elevated temperatures and the character, and especially the depth below the surface, of the habitats which they severally frequent. Doubtless these correlations result, at least in part, from the gradual effects of temperature upon the composition of the body, since they can be determined experimentally (Loeb and Wasteneys, '12); although in just what way they operate, we do not know.

We are chiefly concerned, however, with evidence bearing upon the possibility of a thermal sense, or senses, in *Chiton*. The 'spontaneous' behavior of the gills is perhaps the most significant evidence upon this point, although the nature and variation of the tactile responses are also illuminating. When immersed in sea-water at temperatures of  $15^{\circ}\text{C}$ . or below (down to  $8^{\circ}$ ) the foot of *Chiton* produces a few smooth contractive movements, which are usually not produced at temperatures between  $15^{\circ}$  and  $38^{\circ}$ . The labial palp also moved slightly. The intensity of these movements increased as lower and lower temperatures were employed, down to about  $8^{\circ}$ . The ctenidia contracted in sea-water at  $10^{\circ}$ , and subsequently expanded; there is no regular increase in the vigor or duration of this response with lower temperatures, but it continues clearly down to  $2^{\circ}$ . The abolition of all responses, more quickly the lower the temperature, possibly interferes with the production of other, slower movements which might otherwise result from sensory activation at the lowest temperatures used. With elevated temperatures, not until  $40^{\circ}$  is reached do we find even slight indications of movements, of both foot and ctenidia, resulting from immersion. Above  $43^{\circ}$  these movements did not appear in any form.

These findings are in close accord with the effects of temperature in producing movements among other marine invertebrates. In table 2 data taken from several sources show that the temperatures inducing definite movements indicating response are in general about 15°C, and 35° to 40°C for 'cold' and 'heat,' respectively. This is true of even *Amphioxus*, where Parker ('08) was of the opinion that separate 'cold' and 'heat' senses are demonstrable. In *Ascidia* thermal sensitivity is, however, comparatively great (Hecht, '18). With the exception of *Stylotella*, the animals concerned in table 2 were studied at Bermuda, and at the same season of the year. *Stylotella* has been included in order to show that in a sponge living at a normal temperature corresponding to that of other animals with which it is compared response to a sufficiently high temperature—identical or nearly so with that inducing motor effects in *Chiton*, *Holothuria*, *Amphioxus*, etc.—is clearly produced, although sensory elements are not here (Parker, '10) in any way concerned. The responses obtained at high temperatures do not, in our belief, necessarily demonstrate the operation of differentiated thermoreceptors; they result rather from the general effect of high temperature upon the superficial protoplasm of the animal concerned, leading to increased tactile irritability (Crozier, '15b) and other effects, and may indeed be in some instances due to direct action upon muscle fibers. In *Amphioxus* the motor behavior of the whole animal in removing itself from a localized current of sea-water at 39° is quick and definite, but is not of different character from that to tactile excitation, although evidence from experiments upon differential sensory exhaustion (Parker, '08, p. 440) show that the heat- and tactile-receptive mechanisms are here distinct. In *Chiton*, however, the tests so far cited do not yield conclusive evidence of the presence of heat receptors.

On the low temperature side the results are more encouraging. When the animal was immersed in water at about 12°C. or slightly below, the ctenidia of *Chiton* exhibited a definite contraction of brief duration, after which they expanded. This was true even at 2°, at which temperature tactile responses were very quickly

TABLE 2  
*Summary of data on the thermal responses of certain marine animals. The headings are self-explanatory; under 'Remarks' the methods of stimulation are indicated.*

ANIMAL	HABITAT	NORMAL TEMPERATURE MID-SUMMER	MAXIMAL TEMPERATURE FOR 'COLD' RESPONSE	MINIMAL TEMPERATURE FOR 'HEAT' RESPONSE	NATURE OF THE RESPONSE	REMARKS	TEMPERATURE LEADING TO RAPID DEATH	SOURCE OF DATA
<i>Stylotella heliophila</i> Wils.	Shallow water, near l. w. m. (Beaufort, N. C.)	25 to 28°	(<9°, if at all)	35°	Partial closure of the oscula	Immersion in water current	45° ±	Parker ('10)
<i>Holothuria surinamensis</i> Ludw.	Under seaweeds. (Fairyland Creek, Bda.)	27° ±	15°	31° ±	Retraction of tentacles	Animals slowly heated, or cooled, in water	40°	Crozier ('15 b)
<i>Stichopus moebii</i> Semp.	Muddy bottoms, shallow water (Bermuda).	24 to 27°	12.5°	32°	Cessation of cloacal pumping	Isolated posterior ends heated, or cooled, in water	?	Crozier ('16 b)
<i>Synaptula drifformis</i> (Les.)	Among seaweeds. (Fairyland Creek, Bda.)	27° ±	{ 13° 19° }	{ 43° 40° }	Contraction of tentacles Contractions of whole body	Local applications. Sudden immersion	45 to 46°	Olmsted ('17 b)
<i>Chiton tuberculatus</i> L.	Rocky shores, intertidal (Bermuda)	23 to 37°	12 to 15°	37 to 40°	(see text)	(see text)	44°	Arey and Crozier

Chromodoris zebra Heil.	On sea weeds, (Fairyland Creek, Bda.)	27°±	{ 10° }	35°	Partial con- traction of gills. Protrusion of proboscis, etc.	Immersion in seawater at different tempera- tures	45°	Crozier and Arey
Ascidia atra Les.	On rocks, near l. w. l. (Ber- muda)	27°±	20°	32°	Crossed siphon reflex	Oral siphon allowed to take in hot, or cold, water from a pipette	?	Hecht ('18)
Branchiostoma carribaeum Sund.	Shell sands, shallow water. (Flatts Inlet, Bermuda)	31°	{ 25° } { 15° }	37 to 39°	{ Locomotion Locomotion }	Immersion Local currents	41 to 42°	Parker ('08)



annulled. Immersion in water at 15° frequently led to a few contraction waves appearing on the foot and to movements of the palp, although the ctenidia did not move. Below 8° these movements of the foot were not seen. Between 20° and 38° these movements were absent. The response of the ctenidia to low temperatures is particularly definite and resembles the contraction of the oral tentacles of holothurians (Crozier, '15 b; Olmstead, '17 b), appearing at about the same temperature. These responses are more clearly indicative of thermal receptivity in the strict sense than are those to high temperatures, for below 15° the tactile sensitivity of Chiton quickly diminishes, whereas at 38° to 40° the tactile irritability of the ctenidia, foot, and palp, leading to responses identical with those obtained in the heat treatments, is greatly enhanced for several minutes subsequent to immersion; the augmentation in responsiveness to touch is greater at 40° than at 38°, but at both temperatures sensitivity to touch decreases to below normal after ten minutes and at higher temperatures it disappears more quickly still.

So far, then, we believe that in Chiton there is evidence of something akin to cold reception, but that there is reason to regard the responses obtained upon immersing the animals in warmed sea-water as the result of increased tactile irritability or of some related, non-thermospecific type of irritability.

## *2. Local application of heat and cold*

There is reason to believe that the responses of many animals to chemical influence, for example, and possibly to heat, differ considerably when, 1) a small area of the integument is affected by the stimulation, and, 2) the whole surface is simultaneously exposed to activation. For this reason, and also with the purpose of locating the regions mainly concerned in thermal receptivity (if any should be found), it was necessary to carry out tests in which small portions of the surface of Chiton could be locally heated or cooled. These tests were made in several ways, by the application of small volumes of sea-water at different temperatures or by the use of heated or cooled solid objects. Chiton is

easily employed for such experiments because it can be tested in air, the disturbing influence of a sea-water medium being thus eliminated.

These tests resulted, however, merely in the confirmation of those made by immersing the chitons in sea-water at different temperatures. Even a red-hot needle directly applied to the surface elicited at most but a slight and very local reaction, on the foot and head; the mantle beneath the girdle, the dorsal surface of the girdle, the tegmenta, and the mantle between the plates were quite insensitive. The reactions of the foot and palp, although slight, followed the application quite promptly (within 0.4 seconds). No responses from any region were obtained when a red-hot glass rod was brought within 2 mm. of the animal's surface. A cooled glass rod (at about 5°C.) induced no responses other than those attributable to tactile irritation. With care, a glass rod could be applied even to the gills without leading to a reaction; this was also true with a cooled rod.

Similar results were obtained by the use of test-tubes containing water at different temperatures.

Sea-water adjusted to different temperatures was gently poured in 0.5 cc. volumes from a pipette over different regions of Chiton (in air). At 40°C., local movements of the palp and foot were induced, and a rather indefinite contraction of those ctenidia directly affected; when water at this temperature, or even at 37°, was applied to the dorsal mantle between the plates of an extended chiton, the two neighboring plates were approximated, just as with tactile irritation of this region. Water at 12° led to slight movements of the foot and palp; at 9° to 10°, to prompt local contractions of the ctenidia. Between 12° and 40° no activation was obtained from the general surface.

Although these results are throughout consistent with those outlined in the preceding section, their interpretation presents some difficulties. An attempt was therefore made to separate, through differential exhaustion, the processes of thermal reception from those for tactile and chemical stimulation. The responses to heat and cold indicated that the reactivity of the

various parts of Chiton under the action of these agents follows the relative orders:

dorsal mantle > foot, palp, ctenidia, for heat;  
foot, palp > ctenidia, for cold.

To tactile excitation, the order of reactivity is slightly different (see p. 199), but there exists no adequate criterion for the comparison of the relative sensitivity of the several parts to heat. Only in the case of the dorsal mantle, between the plates, does it appear that thermal sensitivity is relatively enhanced as compared with tactile, since the high-temperature threshold ( $37^{\circ}$ ) seems to be lower than for other regions ( $40^{\circ}$ ) which are superior to the intersegmental mantle in tactile reactivity. The amplitude and vigor of the responses from this region are comparatively slight, however, and little emphasis can be put upon this result. On the basis of their relative distribution, thermal and tactile receptivity cannot be clearly separated.

By differential exhaustion an apparent separation of this kind can, nevertheless, be effected. When water at  $10^{\circ}\text{C.}$  was poured, in 1 cc. portions, from a pipette several times in succession over the anterior ctenidia of a chiton in water at  $24^{\circ}$ , the animal ceased to respond after the fourth treatment; six applications of cool water were made at intervals of three minutes. Very weak tactile responses were then obtainable from the affected ctenidia, although they still did not respond to water at  $10^{\circ}\text{C.}$  In attempting to differentiate between 'heat' and tactile responses, this method of attack fails completely, since, as we have described, when chiton is placed in water at  $38^{\circ}$ , its general tactile reactivity was perceptibly increased, and much more conspicuously so immediately after immersion in water at  $40^{\circ}$ , although tactile reactivity gradually decreases after a few minutes' exposure to this latter temperature. As a consequence of this condition, we are not warranted in speaking either of the separateness or the sensory identity of 'heat' and tactile effects, even though the surface of the foot and the ctenidia did almost cease to respond to touch after they had been exposed to four local treatments, in air, with 1 to 2 cc. of water at  $40^{\circ}$  at intervals of two minutes.

### 3. *Summary*

The evidence we have presented relative to the existence of a temperature sense in Chiton shows that if specific thermoceptors of some sort do indeed occur upon the surface of the animal, they are of a very poorly developed kind. Responses to high temperature, under the various conditions of these tests, cannot be adequately distinguished from tactile effects or even from direct influences upon muscle. The minimal temperature ( $37^{\circ}$  to  $40^{\circ}$ ) eliciting a 'heat response' is very close to the maximal temperature which the chitons successfully withstand, and is even higher than that which induces a distinct effect upon the muscular 'sphincter' about the oscula of *Stylotella* (Parker, '10), where no receptor organs are involved. Although this temperature is identical with that producing heat responses in *Amphioxus* (Parker, '08), it cannot be clearly shown by exhaustion tests—as apparently it can in *Amphioxus*—that 'heat' and tactile receptivity are in any way organically distinct. Only in the case of the intertegmental mantle is there a suggestion of special thermal sensitivity, and here the response elicited is not of a character favorable for analysis. With low temperatures, as with high, the limiting temperature producing perceptible responses in Chiton is probably just outside the range of its normal thermal experience. The 'cold' responses, however, elicited at  $12^{\circ}$  to  $15^{\circ}$ , are of a definite character and may apparently be separated, through differential exhaustion, from purely tactile responses; that they are mediated by special sensory structures remains uncertain, but is possible.

This matter of sensory differentiation is an exceedingly complex one. The fact that isolated cells of the metazoan body (e.g., chromatophores) are capable of excitation by heat, as well as by chemical agents, local pressure, and light (Spaeth, '13), has of course no decisive weight as an argument for 'generalized receptors'; yet the degree of heat (high temperature) effective as a stimulus is in such cases of an order of magnitude comparable to that found effective in the sensory activation of many invertebrates. In comparing the relative sensitivity of different

species, account must be taken of the toughness of the tissue concerned (the delicacy of the respective cell surfaces). This may explain why the delicate, internal, protected surface of the oral siphon of *Ascidia* (Hecht, '18) exhibits a sensitivity to heat and to cold superior to that known for many other animals.

## VI. PHOTIC EXCITATION

### 1. *Effects of light*

*a. Behavior in an illuminated field.*     $\alpha$ . Preliminary experiments. Chitons collected more or less at random and without much attention to size were tested in a qualitative way with reference to their photic behavior. At the bottom of one end of a wooden box, 29 cm. long by 23 cm. wide by 30 cm. deep, there was cut a horizontal slot about 12 cm. long and 1 cm. high. This box was coated on the inside with lampblack suspended in turpentine, giving an approximately dead-black finish. A rectangular glass jar containing sea-water to a depth of several centimeters was placed inside the black box, within which it fitted closely. Chitons were put in the glass jar, the box covered, light admitted (or directed) through the slot, and the subsequent movements of the animals determined.

With diffuse sunlight twenty-one experiments upon twenty individual chitons gave this result: 6 did not move at all during the course of the test (lasting about one hour); 1 oriented a few degrees away from the light, while 13 animals made definite progress toward the light, irrespective of their original orientation in relation to it. These chitons were probably all of average size or larger. In some instances they were allowed to become fixed to the bottom of the aquarium with their anterior ends toward the light, in other cases they were placed with long axis perpendicular to the light, in still others deliberately headed away from it or quite at random. The nature of the result in these experiments will be evident from the following record:



*Experiment 1.*

- 11:15 A.M. A chiton placed with long axis parallel to the slot admitting light, distant 13 cm. from it.  
11:19 A.M. Oriented toward the light.  
11:20 A.M. Began moving forward. At first moved in a diagonal direction, until the girdle touched the side wall of the container; it then turned further and moved directly toward the light.  
11:24 A.M. Stopped, half-way toward the slot.  
11:30 A.M. Began moving again.  
11:34 A.M. Reached light end and began climbing end wall.  
11:39 A.M. All except posterior quarter attached to end wall of container. Stopped.  
11:43 A.M. Began again and moved until all of body was on vertical end wall. Turned until body axis was parallel to water line, where, just submerged, it lay directly over the slot.

*Experiment 2*

- 11:45 A.M. Same chiton as in experiment 1, placed transversely to the light, but with other side illuminated, and 26 cm. from the light slot.  
11:48 A.M. Began turning away from the light.  
11:50 A.M. Had rotated away from the light,<sup>8</sup> then back toward it, through an angle of more than 270°.  
11:54 A.M. End had come in contact with side of container. Animal now began to climb. No forward progress toward the light.

*Experiments 3 and 4*

In two further chitons tested in this way, orientation was in one case direct, beginning almost at once; in the other it required 29 min. (involving a preliminary turning through 45° away from the light). Both animals made definite progress toward the light.

These tests indicated in a general way the presence of a definite, though sluggish, positive phototropism, with reference to diffuse light.

With direct sunlight, reflected horizontally from a mirror, three individuals oriented promptly and move directly toward the light, two oriented toward the light and then away from it, four individuals immediately oriented more or less away from the light, and two did not move at all. This result obviously required further analysis; it might have been the outcome of a general illumination of the whole aquarium or might have ref-

<sup>8</sup> Note this apparent persistence of a turning tendency once established.

erence more specifically to some definite peculiarity of behavior. These tests were made with chitons of relatively large size (6 to 8 cm.) in which the shell valves were probably more or less eroded, although at the time no special note was made of their condition.

β. Analyses of responses to general illumination. The foregoing section indicates the somewhat obscure relations, with respect to phototropism, discovered in random samples of the chiton population. The younger individuals, especially those less than 2 cm. long, live in dark situations. When stones bearing them are turned over, the chitons creep rapidly to the under, dark side. Not until a length of 7 to 9 cm. is attained does *Chiton* occur with any frequency upon illuminated rocks. If chitons from habitats representing these two divergent extremes are compared, it is found that in ordinary sunlight the larger ones are photopositive, the younger ones photonegative. Their orientation is precise, definite, and without 'trial movements.' There are, however, certain complications in the mode of orientation which will be fully considered on a later page. The following test is typical:<sup>9</sup>

LENGTH OF INDIVIDUAL	HABITAT	PHOTIC BEHAVIOR
cm.		
1.0	Under stone on a sandy beach, south side Darrell Island. (No. VI, 119.B) <sup>9</sup>	Consistently photonegative to the weakest daylight used.
2.1	Same locality. (VI. 119.C)	Photonegative to direct sunlight; photopositive to weak diffuse light, and to twilight.
3.5	In a pocket at the mouth of a cave, north shore Long Island. (VI. 122.1)	Photonegative to direct sunlight; photopositive to light from a north window 10 ft. away.
5.0	Same locality (VI.128.9)	Same
7.2	On an approximately horizontal rock, exposed in the sun, north shore Marshall Island. (VI. 140.2)	Photopositive to diffuse daylight; photopositive to direct sunlight from a cloudless sky
8.3	Same locality. (VI. 140.4)	Same.

<sup>9</sup> The specific animals bear definite numbers given to them in the field notebook. In a subsequent report on the ethology of *C. tuberculatus* the necessity for this will be made apparent.

These six specimens illustrate a correlation which is universal in our experience: the youngest chitons are found in dark-situations, the older ones in the light; these two groups are, respectively, photonegative and photopositive to ordinary sunlight; animals of intermediate size are photopositive to weak light, photonegative to stronger light, and the character of their normal photic environment (typically, in horizontal crevices near the mouth of caves, and in other non-brilliantly illuminated spots) is completely correlated with this behavior. There is usually a region of intensities of ordinary daylight within which an animal of intermediate size may be either photopositive or photonegative in different tests—a region of seeming indifference to light. The largest chitons are usually quite indifferent to weak, diffused light. The actual distribution of the chitons of different sizes in the field shows in a most convincing manner that this differentiation in photic responses is not a matter of adaptation to environmental circumstances, but is on the contrary based upon structural changes determined with advancing age. Note, for example, the following instances in which an individual photonegative to sunlight (as found by test) occurred on a shore where no deep caves were available, nor any large stones under which it might hide.

VI. 111. (Apl. 4, 1918). North shore of Hawkins Island; a more or less horizontal shelf of rock, 1 foot beneath high water mark; nine chitons in a closely compacted group, in the zone of *Modiolus* and barnacles. Eight of the chitons with eroded valves, forming a fairly close match with the color of the exposed rock; in sunlight; these chitons 6.5 to 8.8 cm. in length. One chiton, however, was a ♂, 4.5 cm. in length, the valves greenish, very slightly eroded; it was located under another individual (♂, 6.8 cm. long), which completely concealed and sheltered it from the light.

VI. 140 (Apl. 22, 1918). Northwest shore of Marshall Island. In a small pocket in the rock four chitons of 7.2 to 8.7 cm. were found; of these the shells were eroded and bleached. Under one of them a fifth specimen, 2.8 cm. long, blue-green in color, valves uneroded.

*b. Results of partial illumination of the body.* α. One side of the body illuminated. Chitons were adjusted in the apparatus shown diagrammatically in figure 13; they were so situated, directly under the vertical partition, that either the right or left

side was in comparative darkness, the other side in the light. Diffused sunlight, employed with chitons of medium to large size, induced responses of a variable kind.

*Experiment 1.* Two chitons introduced. One moved partly toward the dark side, and subsequently, in the course of 45 min., moved back into the center of the light compartment. The other one rotated through  $180^\circ$ , its anterior end passing through the dark side and then orienting into the light.

*Experiment 6.* Two chitons used. One began to move at once; oriented  $90^\circ$  into the dark, and moved so that almost the whole of its body was in the dark compartment; after 5 min., it turned through  $180^\circ$  and moved straight out into the light. The other one within a

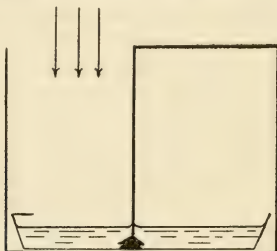


Fig. 13 Sectional view of apparatus for the partial illumination of a Chiton: a shallow pan fitting the bottom of a box with blackened walls; one half of box open to receive light, as indicated by the arrows, the other half covered and separated from first half by a blackened vertical partition extending into the sea-water, that nearly fills the pan; horizontal overhang from the lip of the pan exposed to light reduces reflection.

few minutes oriented weakly into the light, halted 1 min., and then oriented anterior end back into the dark, so that half of its body was on either side of the partition.

In 15 individual tests of this nature,

4 chitons turned and moved directly into the light, where they remained.

0 chiton turned and moved directly into the dark.

4 chitons turned and moved into the dark, then into the light.

0 chiton turned and moved into the light, then into the dark.

4 chitons turned directly into the light, but did not creep.

0 chiton turned directly into the dark, but did not creep.

1 chiton oriented into the light, then into the dark.

2 chitons gave no response during the time allowed for the experiment.

β. One end of the body illuminated. Similar tests were made in which either the anterior or the posterior end of the animal was illuminated. Numerous experiments of this sort were also made in the field. In brief, chitons upon sunlit rocks were found to move into the light when either the anterior or the posterior end had been shaded. The locomotion subsequent to illumination of the posterior half only, by means of bright sunlight, was in a posterior direction; usually, before the chiton had moved completely into the light, it executed a turning movement. The readiness with which backward creeping, for distances of several centimeters, may be resorted to, is worthy of remark.

γ. Analysis. The predominating movement in these tests is photopositive. The peculiar variations observed remain to be explained. This can be done, as in the case of orientation by general illumination, through consideration of the size and habitat of the individuals and of the character of their periostracum and tegmenta. This analysis agrees in its results with that previously given. The small chitons, less than 2 cm. in length, move into the shadow when their surface is half illuminated. They creep backward with greater readiness than do the large ones.

These experiments show that with light approximately vertical in direction the effect of partial illumination of the body is such as to parallel completely the result in orientation to horizontal light.

## 2. *Differential sensitivity*

*a. Shading.* An attached chiton, undisturbed and at rest, tends to lift the girdle from the substratum, either along its whole circumference or else in one or more local areas. If a raised portion of the girdle be shaded, there results a relatively quick and smooth lowering of the girdle to the rock or other surface. This response occupies about two seconds; its vigor depends upon the original distance of the girdle from the substratum. After a little time (about ten seconds) the part contracted is relaxed to its original condition. The speed of reaction and the time for recovery vary considerably in different animals. In some cases a single stimulation resulted in the



girdle being closely applied to the substratum for a long time (save at the anterior and posterior ends, involved in respiration). In other instances five to nine shadings and responses were obtained, after which the response grew weaker until it finally disappeared. When the response became very weak, or imperceptible to a single shading, two or more shadings in fairly rapid succession were still effective in producing a reaction from the girdle. Associated with depression of the girdle is a pronounced contraction of the gills in animals shaded dorsally.

Chitons resting upon their dorsal surface and shaded ventrally gave also a pronounced response. Some individuals were very sensitive, coming from a fully extended condition to complete rolled-up closure as the result of a slight decrease in the illumination; if already partially rolled up, a general shading induced still further, but still incomplete, curling of the body, after which the chiton returned to its original condition. The response became fatigued after seven to sixteen trials. In good reactions of this type, five seconds were required for the curling up of the shell, fifteen seconds for its recovery. To successive shadings the curling-up process becomes more complete; the time required for this effect varies directly with the size of the animal, as seen in the following notes (table 3); some animals are decidedly less reactive in this way than others, however. In *Holothuria* (Crozier, '14) conditions of similar import are known.

The reaction to shading of the ventral surface comprises longitudinal bending, contractive movements of foot and head, inward curving of the edge of the girdle, and contractions of the gills. The time occupied by the response of the ctenidia ('reaction time') to a single shading at the posterior end of the animal is also proportional to the length of the Chiton (table 3).

The response to shading is due to a decrease in the intensity of light in the visible region of wave lengths. The interposition of a glass plate between a chiton and the source of light led to no reaction, and the shading response was easily elicited through a considerable thickness of glass. Glass ray filters transmitting restricted portions of the spectrum were placed between chitons and a source of sunlight (which induced shading responses),

and then the effect of cutting off the colored light was determined. The transmitting regions of the spectrum for the color filters used are given in table 4. These values are of course not very precise.

The red filter transmitted light that seemed about twice as bright as that coming through the blue. Nevertheless, the reactions obtained upon shading chitons through these filters

TABLE 3

INDIVIDUAL		TIME REQUIRED TO EFFECT ROLLING UP OF THE SHELL BY REPEATED SHADINGS OF THE AN- TERIOR END	'REACTION-TIME' OF POSTERIOR CTENIDIA TO SINGLE SHADING
Number	Length		
	cm.	seconds	seconds
1	3.0	5	
2	5.5	10	
3	9.0	15	
4	3.5		1-2
5	6.0		2-3
6	8.9		4-5

TABLE 4

*Regions of spectrum transmitted by certain color filters*

COLOR	LIGHT TRANSMITTED mμ	RANGE mμ
Red.....	690-634	56
Yellow.....	690-605	85
Green.....	589-508	81
Blue.....	523-450±	73

were sufficiently clear cut to be of some use. Responses produced by cutting off the light coming through the green or the blue screen were equal in amplitude to those obtained in sunlight; whereas the red and yellow lights, when occluded, led to quite obviously weaker responses. This is also the case in the shading reactions of the shore barnacle (Crozier, '15 b, p. 273; the same light filters were used).

Chitons of all sizes (ages) and from every type of habitat give

precise responses of the character described when the light intensity is suddenly reduced.

*b. Increased light intensity.* Chitons from medium to large size, under water in aquaria placed in direct sunlight, give also a response to increased illumination. This response is not evident except in bright sunlight. It consists in a depression of the girdle similar to that induced by shading. The response is, however, never of such quickness, vigor, or completeness as that to shading. In smaller chitons, or with larger ones in diffuse light, the reaction to increased illumination (if present at all) is so slight as to escape detection. At its maximal development, it comprises a local depression of the girdle to the substratum, and does not involve a 'suction reflex' of the whole animal, such as is induced by a very slight decrease in illumination. The threshold of sensitivity for increased illumination is, indeed, very much higher than that for decreased.

It should, however, be noted that if a large chiton is shaded dorsally the girdle may not be much elevated again for some time, provided the state of lowered intensity is allowed to continue. On removing the shadow, the girdle is again elevated. If the intensity be slightly decreased the girdle of a large chiton may be lowered rather slowly, so that after two seconds the shadow may be removed before the girdle has been completely depressed; the movement of the girdle may then cease abruptly with the incidence of the more intense light.

A similar response is evidenced upon the ventral surface of the animal. If the light be suddenly increased (to direct sunlight) about two seconds subsequent to the beginning of the 'curling-up' response induced by a shading, this response sometimes ceases abruptly; the chiton may or may not proceed then to straighten out.

The effects of increased illumination are more conspicuous in lamplight at night. Clear indications have been obtained of a specifically higher photic sensitivity at night as compared with daylight hours. A discussion of this matter awaits further investigation.

### 3. *Distribution and nature of photoreceptors*

*a. For illumination.* We have described thus far the responses obtained from chitons submitted to photic excitation involving the edge of the girdle, a large portion of the surface, or the whole surface of the animal. There remain to be considered the distribution and variety of the photoreceptors.

A chiton from which the girdle has been completely removed is still oriented by light. This fact, taken together with the known histological structure of the sensory organs in the shell plates, suggested that the surface of the shell might contain photosensitive organs. Chitons were rigidly clamped in the way already described in discussing tactile stimulation (p. 200), and allowed to write their contractions kymographically. Tested in air, the shell was found to be sensitive to moderate faradization. When so stimulated, the animal undergoes a pronounced general 'curling up,' which ceases when the current is interrupted. A similar result follows the application of a spot of light.<sup>10</sup> By this method it can also be shown that the shell plates are sensitive to shading. They do not appear sensitive to increased illumination.

These results, together with those obtained in orientation experiments, furnish adequate proof that the 'shell eyes' of chiton are indeed sensitive to light. We did not attempt to localize the minimal areas sensitive to light, but the illumination of one-eighth of the surface of an anterior or posterior valve with an intense spot of light is sufficient to induce a good response.

In the genus *Chiton* 'extrapigmental' eyes are lacking (Moseley, '85; Plate, '99; Nowikoff, '09), the 'intrapigmental' eyes being, however, plentifully distributed over the surfaces of the valves according to a definite pattern; their locations are well shown in eroded shells. They are most abundant in the anterior and posterior valves, but occur on all the shell plates.

The 'eyes' are not the only parts sensitive to light, however; the ventral edge and the dorsal surface of the girdle indicate by

<sup>10</sup> This method of investigation permits quantitative work of a relatively precise kind; but in the present paper we are concerned mainly with qualitative effects.

movements a local sensitivity to illumination, as likewise the anterior edge of the proboscis probably does. Heath ('99, p. 579; sep. p. 4) thought the proboscis of *Ischnochiton* to be sensitive to light.

In the adult *Chiton* there are no detectable cephalic eyes, although in some small species the larval 'eye' may persist into adult life (Heath, '04 b). The larva of *Ischnochiton magdallensis* is positively phototropic (Heath, '99, p. 637; sep. p. 62).

*b. For shading.* The edge of the girdle and the tegmental surfaces of the valves are sensitive to decrease in light. In the former case the nature of the receptors is obscure; in the latter case they may or may not be identical with the organs sensitive to the constant action of light. The minimal area which must be shaded in order to effect a response is very small. The shadow of a fly 6 feet distant in moderately bright sunlight induces violent shading responses. An opaque spot 2 mm. in diameter on a glass plate at a distance of about 2 cm., casts sufficient shadow upon a valve to lead to pronounced reactions. The threshold of sensitivity is very low, an almost imperceptible decrease in light intensity being quite effective.

The arrangement of the aesthetes in the tegmentum is such that many microaesthetes accompany each megalaesthete; it might be supposed that the microaesthetes are of different receptive value, but no proof for this can be given.

The physiological distinctness of the shading receptors on the ventral edge of the girdle is shown by the fact that their complete exhaustion by repeated activation does not interfere in the least with tactile responses.

*c. For increased illumination.* The great sensitivity of chiton to shading and the poorly developed character of the responses to increased illumination make adequate experimentation very difficult. The edge of the girdle is the most sensitive part; probably the surface of the proboscis and palp are also sensitive. Nothing more can at present be said under this head.



#### 4. *On the theory of phototropism*

a. *The orienting stimulus.* In a previous article (Crozier and Arey, '18) we have given some discussion of this matter, and need refer here merely to the main conclusion. In young individuals the simultaneous exhibition of a precise reaction to shading, and no such response to increased illumination, is thoroughly inconsistent with the idea that negative orientation by light is induced by stimulus derived from an increase of illumination, as such (Crozier, '14, '15 b). The further continued presence of this shading response in older chitons under conditions in which they are photopositive, together with the fact that any reaction to increased light intensity which these older chitons did exhibit was clearly of a negative character, is of the first importance theoretically. It completes the cycle of qualitative proof that the constant action of light, not change of intensity, is the causal stimulating agent in photic orientation.

Two further types of experiment may be cited in this connection.

A chiton of medium size orienting away from the light is purposely shaded on the side to which it is turning. Light falling at an angle of about  $45^\circ$  is used in the experiment. The chiton reacts by depressing the girdle, as usual, and swings somewhat away from the shaded side. By repeated successive shadings in this way, the chiton may be made to move for a time at an angle with the line of the incident light, although after it becomes exhausted for shading it completes its orientation in the usual way.

A chiton completely exhausted by shading orients without delay away from or toward the light, depending on the age of the animal.

Both types of experiment result in behavior agreeing with the conclusion above stated.

b. *The determination of positive and of negative orientation.* In an earlier section of this paper we have called attention to the fact that at a length of about 5.5 cm. (for the Chiton population in Great Sound) the shell begins to show noticeable erosion,

This erosion continues with increasing severity until natural death supervenes. By its ravages the canals containing the aesthetes are laid bare and the sense organs therein contained are destroyed. The empty canals are visible under a hand lens. This phenomenon was noted by Plate ('01 a, p. 383) in connection with forms possessing large extra-pigmental eyes. At a length of 7 cm. the erosion is quite pronounced; the shell is also frequently covered with adventitious organisms. We have shown the organs of photoreception concerned in orientation to be located in the tegmenta of the valves. There is thus a pronounced correlation between the development of positive phototropism toward daylight and the erosion of the shell, for animals less than 7 cm. length are rarely found completely exposed to sunlight.

There are a number of instances in which animals alter the sense of their phototropism depending upon the intensity of the light. It is in general the rule that this alteration is one from a photopositive condition in weak light to a photonegative condition in strong light, rarely the reverse. The general rule holds in the case of *Chiton*, and in other forms exhibiting general integumentary sensitivity to light. The indifferent point dividing the range of light intensities into a lower range, exciting positive orientation, and a higher range, inducing negative phototropism, shifts in *Chiton* toward the upper limit as age (and erosion) advances.

Hence it would appear that in this animal the excitation of a smaller number of sense organs (in the eroded chitons) is equivalent in stimulating power to the action of a lower intensity of light upon a larger number of sense organs. This is further evidence pointing to the constant action of light as the source of the orienting stimulus. No case of this kind has previously been examined. It will be interesting to make a further study of this matter, in relation to temperature, for example.

*c. The method of orientation.* In *Chiton tuberculatus*, especially in older, unevenly eroded animals, there are to be found traces of a condition, more plainly evidenced in *Ischnochiton purpurascens*, which is at first sight anomalous. Sometimes an

animal will orient precisely away from the light, and then move caudad toward the light for a distance of several centimeters. Close inspection usually makes it evident in these cases that the posterior valve is more deeply eroded than the anterior valves. Its sensitivity is therefore less. Similarly, a few animals were noted in which photopositive orientation was succeeded by backward creeping, i.e., away from the light, owing presumably to the higher sensitivity of the anterior valve. There is evident in these reactions a type of local response, rather than of unitary behavior, which is highly instructive and deserves further study.

In *Ischnochiton* the behavior above referred to is somewhat similar to that just described, as this amphineuran creeps posteriorly with considerable freedom. Horizontal light parallel to the long axis, incident upon the anterior end, induces the animal frequently to creep backward for several centimeters.

In chitons relatively uneroded or evenly eroded the method of orientation is direct, diagrammatic, as it is in most mollusks. In a large individual the process is so slowly carried out, unless very bright light is used, that its details may be carefully examined. The results of such examination are not merely consistent with the theory of Loeb regarding animal phototropism, but furnish valuable evidence in support of this view. The behavior of *Ischnochiton* and of unevenly eroded large individuals of chiton is of particular importance in this connection.

##### *5. Bionomic correlations involving photic behavior*

Many writers have considered the photic reactions of animals in the light of their normal conditions of life, coming usually to the conclusion that these reactions are highly adaptive, and hence that they have been determined through natural selection (Mast, '11, chap. 13). In spite of its traditional sanctity, the presentation of this view involves an inversion of logic, a veritable somersaulting, which is to the last degree unconvincing. That the modes of response which specific organisms are found to exhibit are individually correlated in an appropriate way with

the conditions of existence, no one of experience will be prepared to deny; there are also, of course, modes of reaction under conditions artificially imposed which are important from this standpoint. It is rather the harmonious complexity of these individual responses which presents in reality the difficult problem and the one of importance. The behavior of *Chiton* is most illuminating in this respect.

In an earlier chapter we have briefly touched upon the coördinations between size, habitat, coloration, and photic orientation in the *Chiton* population. The main fact appears to be that as *Chiton* gets older it moves out into more open situations. The other correlations follow automatically in the wake of the changing sense of the animal's phototropism. This change is due to a gradual shifting of the 'indifferent point' of light intensity separating the region of lower intensities, leading to photopositive behavior, from the region of higher intensities, leading to photonegative movements. We have shown that the principal factor involved in this alteration is the erosion of the tegmenta of the valves (bringing about the destruction of the photosensitive aesthetes).

It remains to discover what it is that produces the erosion of the shell. Comparative studies of differing environments frequented by *Chiton* are now in progress and should ultimately afford a quantitative answer to this question. The erosion of the shell is general among the chitons of large size. Plate ('01 a, pp. 381-3) supposed it to result from 'wave action' and the mechanical effect of sand. In *C. tuberculatus* it would seem that the periostracum is in part eroded as a result of chemical action of the water, combined with the failure to continue its secretion upon older parts of the shell; as well as to the activity of barnacles, algae, and other organisms which settle upon the valves.<sup>11</sup> In different localities it would seem that these two factors are of

<sup>11</sup> Barnacles live upon the valves of a chiton until they have formed two or three distinct growth lines. The firmness with which they are attached to the shell plates of *Chiton* depends upon the degree of the original erosion. In chitons of medium size the barnacles usually are not very firmly attached; after death they drop off and leave no scar. On older chitons (seven to nine years) the dead remains of barnacles adhere for some time.

varying relative importance. The general feature which is the most significant, however, is the correlation of slight exposure with incipient erosion. A study of over 1500 individuals has shown the invariable completeness of this correlation. The amount of exposure is, in part at least, determined in a purely mechanical way. The smallest chitons, dwelling at the very upper limit of the tide, under loosely piled small stones, become after a year or two too big to fit into the crevices there provided. The food supply is also insufficient. They therefore come to inhabit stations further below high-water mark. The occurrence of other organisms is here more abundant, and there are additional factors making for more ready erosion.

In this way the history of a chiton can be followed in a fashion which shows that although its habitat is determined by its behavior, the reverse is not apparently the case. Hence the logical inversion to which reference was made in the first paragraph of this section. It is also evident that the modifications in *Chiton's* behavior and appearance, its occurrence in groups, and the probably advantageous correlations in this way resulting, are determined in a catenary manner. The original position (habitat) of the young individual is determined by the tropisms of the larva, in their turn determined by the inherited chemical composition of the egg. The lack of active wandering movements (p. 178; Plate, '01a, p. 509) in the older individuals is important, because opportunity is in this way made for the full operation of the influences in particular circumscribed habitats, and thus for the development of homochromic elements in the coloration of the chitons and for concomittant phases of progressive erosion of the shell.

The chitons as a whole are known to be photosensitive and to reside in general in dark situations (Cooke, '95, p. 400). Heath ('99) observed that a number of chitons were nocturnal in their habit, "withdrawing into some shaded position upon the approach of day," some species remaining out on their feeding grounds "only when the day is foggy or dark." The larva of *Ischnochiton magdalenensis* is positively heliotropic, the adult negative (Heath, '99). It would be of some interest to study a variety of



species from the standpoint we have developed in this paper (Plate, '01 a, p. 509). *Ischnochiton purpurascens*, *Acanthochites spiculosus*, and a species of *Tonicia* we find to be photo-negative to light of all intensities. The deep-sea solenogastres may also be photosensitive, although the only observation known to us on this point (Heath, '04a, p. 461) is not decisive.

## VII. CHEMICAL EXCITATION

### 1. *Reactions to various substances*

The surface of the soft parts of *Chiton* is sensitive to a wide variety of chemical excitants. We are concerned, in the first place, to discover something of the nature of the process of chemical excitation by electrolytes. For this purpose experiments are cited in which, unless otherwise stated, approximately 0.5 cc. of solution was locally applied to the surface of chiton, tactile stimulation on the part of the stream being avoided. The chitons were tested in air. After each response, the stimulating fluid was washed away by a gentle stream of sea-water before proceeding to any further tests.

*a.* It was attempted to arrange certain salts in the relative order of their stimulating power. The alkaline cations K,  $\text{NH}_4$ , Li, and Na, and Ca and Mg, were compared through the effects of their chlorides, in 5/8 M solutions (made up in rain-water). The mouth, the sole and the edge of the foot, the gills, and the ventral surface of the girdle were stimulated in various individuals. The comparative efficiency of the different chlorides was judged on the basis of their average effects upon all the parts activated. Since it may be supposed that an effectual subjective factor entered into these judgments, we will give rather full notes of the experiments.

*KCl:* Very strong stimulation in all regions, even on the ventral surface of the girdle, which bends inward toward the source of excitation. All the responses very sharp.

*$\text{NH}_4\text{Cl}$ :* Also gives pronounced contractive responses, but not so excessively strong as with KCl, nor of such long after duration. No responses from the girdle.

*LiCl*: Much as with  $\text{NH}_4\text{Cl}$ , but reactions noticeably weaker; responses not initiated so quickly, nor so completely carried out.

*NaCl*: Responses in the mouth region extremely weak; on the edge and the sole of the foot, still weaker; the gill responses possibly as decided as with  $\text{LiCl}$ . The edge of the foot moves at first toward the stimulating liquid, then away.

*CaCl<sub>2</sub>*: No reactions from the gills; on the mouth region, and the edge and sole of the foot, stronger responses than with  $\text{NaCl}$ .

*MgCl<sub>2</sub>*: The gills were raised toward the source of application; weak negative response from the sole of the foot; the edge of the foot at first pushed locally toward the fluid—as with  $\text{NaCl}$ —then away if the application were prolonged. In the mouth region, fairly strong contractions.

From these findings it appears that the cation order of decreasing stimulating power is



Subsequent experiments with more dilute  $\text{CaCl}_2$  and  $\text{MgCl}_2$  solutions, isosmotic with sea-water, showed that to the former salt practically no responses were given except at the mouth, while to the latter no responses were given save the positive out-pushing of the ctenidia. These bivalent cations are markedly less efficient in sensory excitation than are the monovalent alkaline cations. The curious 'positive' reaction of the gills to  $\text{MgCl}_2$  solutions is similar to that given by the podia of holothurians (Crozier, '15 b).

b. The neutral salts of potassium— $\text{KCl}$ ,  $\text{KBr}$ ,  $\text{KNO}_3$ , and  $\text{KI}$ —were compared, at 5/8 M concentration as in the previous tests. The local reactions elicited were very violent in all parts of the body; but in no place were the animals stimulated to roll up. The ctenidia responded by contracting until curled in a tight circle against the dorsal wall of the gill cavity; at the same time the side of the foot was moved far away, laterally, thus exposing the gill cavity more widely, while the girdle was rolled over and inward so as to cover the ctenidia. After a brief interval the foot relaxed. These responses were of variable duration, depending upon the amount of the stimulating solution applied.

For purposes of comparison, 'reaction times' were measured, covering the interval from the contraction of the gills until the moment they were judged to be completely relaxed.

The results obtained in this way may be illustrated by the following sets of data:

SALT SOLUTION	TIME OCCUPIED BY THE GILL REACTION		
	SERIES I	SERIES II	SERIES III
	<i>minutes</i>	<i>minutes</i>	<i>seconds</i>
KCl.....	5.0	3.7	
KNO <sub>3</sub> .....	1.7	1.5	30
KBr.....	1.0	1.0	35
KI.....			20

KCl was plainly more stimulating than the other salts. After numerous attempts to graduate the other three under various conditions as to quantity of solution and quickness of application, in the same and in different animals, the series

$$\text{KCl} > \text{KNO}_3 > \text{KBr} > \text{KI}$$

was chosen as the most satisfactory.

c. Successive dilutions of several representative substances were employed to stimulate various regions of chiton's surface, with the object of establishing the respective limiting dilutions effective in excitation. Sea-water was largely used as the solvent in these tests, since it is the normal fluid medium for chiton; moreover, as we shall see presently, the surface of the animal is reactive locally to osmotic conditions differing from the normal.

*KCl*: To KCl solutions more concentrated than N/16, made up in sea-water, all of the ventral portions of Chiton are reactive.

N/16	All portions respond except the girdle.
N/32	No response from the sole of the foot.
N/48	Good reactions from the mouth region; the gills sometimes fail to react.
N/64	Gills fail to respond.
N/80	Mouth response good; edge of the foot weak.

- N/112 Edge of the foot fails; the palp is sensitive on its edge and bends first toward, then away from, the stimulus.
- N/160 Faint responses from the mouth region.

From one animal, considerably more sensitive than the rest, faint mouth-region responses were also obtained at dilutions N/224 and N/256; this was very exceptional.

*KOH*: The dilutions tabulated are here only approximate, owing to the precipitation of calcium and magnesium hydrates at concentrations above 0.012 N  $\pm$  KOH (Haas, '16).

- N/10 Reactions produced on all parts except upon the ventral surface of the girdle (mantle edge).
- N/50 to N/125 Same.
- N/250 to N/375 The edge of the foot is but slightly sensitive.
- N/500 No responses from the foot; reaction at the mouth just perceptible; gill responses of fair intensity.
- HCl*: N/10 No reaction from the ventral surface of the mantle edge.
- N/50 to N/425—responses from all parts, save the mantle edge.
- N/500 No reactions from the foot; very faint responses from the mouth; gills respond more weakly still, but with fair sensitivity.

Several organic acids were also tested. At M/10 concentration, in rain water, tannic, malic, lactic and acetic acids produced reactions from all parts, including the mantle edge. M/100 solutions of the same acids made up in sea-water also induced responses, but not from the mantle edge; these acids were compared by noting the 'recovery time' of the gills when stimulated with them (as in the case of salts); the measurements obtained were:

- Tannic, 35 sec.  
Malic, 20 sec.  
Lactic, 12 sec.  
Acetic, 8 sec.

*Picric acid*: made up in sea-water, produced very powerful reactions everywhere, at concentrations above M/150.

- M/250 Responses of the same character, but weaker.
- M/500 to M/750 The edge of the mantle fails.
- M/1000 The foot still sensitive, especially along its periphery.
- M/1200 Gills fail; foot and mouth give good reactions.
- M/1500 Foot fails; mouth alone responds, but smoothly and evenly.

*Cocaine hydrochloride, atropine sulphate, and nicotine* at M/100 concentration in sea-water stimulated very well the mouth, gills, and the sole and edge of the foot.

*Chloretone* M/200 in sea-water also stimulated these parts; but *Urethane* under the same conditions did not.

*Curare* in saturated sea-water solution, gave rise to good, but not pronounced responses.

*Urea*. A M/10 solution, in rain water, did not lead to any clear response on the part of the foot, and gave but weak responses from mouth and gills.

*Ethyl and methyl alcohol* solutions 10M, in rain-water, evoked reactions from all parts save the mantle edge. With the methyl alcohol the responses were slightly stronger, the gill retraction enduring about twice as long (about 20 sec., as compared with 8 to 10 sec. for the ethyl); this may have been due to impurities; the ethyl alcohol was especially pure. Full strength amyl alcohol induced responses as with the two previous types, but considerably stronger movements resulted.

It has been of interest to inquire if the surface of *Chiton* is generally sensitive to sugars or to 'sweet' substances as a class. In view of the usually high value of the limiting stimulating concentration for sugars, it is necessary to consider the following observations in the light of the subsequent experiments upon osmotic excitation:

*Maltose*: solutions M/3 or M/10, in sea-water, produced no clear responses from any part.

A 1M solution in rain-water gave responses from mouth and gills only.

*Sucrose*: 1M, in rain-water, led to no responses from the mouth region, but gave good reactions from the ctenidia.

M/3 in sea-water, gave general reactions from all parts.

M/2, made by adding 1 volume of 1M in rain-water to 1 volume of sea-water, led to fair responses.

M/3, made by adding 1 volume of 1M in rain-water to 2 volumes of sea-water, induced no reactions at all.

*Lactose*: M/2 in rain-water gave strong responses everywhere except at the mantle edge.

d. To a variety of more obvious 'irritants,' the whole soft ventral surface of *Chiton* reacts powerfully. Thus,  $\text{H}_2\text{O}_2$  (3 per cent) induces strong movements in all regions. Ether, chloroform, carbon bisulphide, aniline oil, and oils of cassia, juniper, cloves, pennyroyal, thyme, bergamot, and origanum, when applied as a drop of the raw substance or in the form of a satu-



rated extract in sea-water, induced good reactions from all parts, including the girdle, which reacted by bending and twisting away.

The order of sensitivity of the several parts of the surface was made out to be:

gills > head > edge and posterior end of foot > sole of foot > ventral girdle surface > dorsal girdle surface.

e. Osmotic excitation of the general ventral surface of Chiton was investigated by means of dilutions of sea-water.

*Three parts sea-water + 1 part rain-water:* gave responses only upon the lips.

*Two parts sea-water + 1 part rain-water:* no response from the mantle edge, but other parts react weakly.

*One part sea-water + 1 part rain-water:* no response from the mantle edge, good responses from other parts.

*Rain-water:* sole of the foot puckers away strongly; gill response very active; even the girdle reacts, and the shell tends to roll up.

*Sea-water concentrated by evaporation to one half its original volume* gave good reactions on all regions.

The same upper limit of osmotic stimulation appears in the effects of glycerin solutions:

*Glycerin:* in sea-water solution,

5M Strong reactions everywhere, even from the mantle edge.

3M Mantle edge fails; pitting of the foot away from the fluid is deep and local.

2M Same, but weaker responses.

1M Ctenidia give a faint response, but only occasionally; mouth region still sensitive; very doubtful responses from the foot.

M/2 Faint response from the lips; ctenidia doubtful.

## 2. The mode of excitation by solutions

The observations above detailed require analysis from several points of view. We shall deal first, briefly, with the evidence they contain relative to the method of activation by solutions.

a. It is clear that 'osmotic sensitivity' is possessed by the soft superficial tissues of chiton. Whether this depends upon the activation of 'tactile' or other end organs or upon the stimulation of chemoreceptors proper we cannot at first entirely

decide. The comparative distribution of tactile and of osmotic sensitivity is nevertheless suggestive in this connection. We have seen that for stimulation by gentle contact the reactivity of the several regions of chiton's surface was as follows:

head = ctenidia > edge of foot > girdle > sole of foot;

while for irritants, such as essential oils, the order of reactivity for the same parts was:

ctenidia > head > edge and posterior end of foot > sole of foot > girdle.

To local osmotic disturbances the sensitivity of these areas appeared, on the basis of the experiments with dilutions of sea-water, to be related in the following sequence:

head > ctenidia = foot > ventral edge of girdle.

So far as these responses go, they indicate that the receptors concerned in osmotic excitation are distinct from those concerned in tactile reactions, from those concerned with responses to irritants, and (as seen in a following section) from those implicated in chemical excitation, but the evidence is not conclusive.

The sensitivity of the proboscis ('head'), especially of its peripheral edge, is probably concerned in determining the relative immobility of chitons in exposed places at the period of low tide; the same, to a lesser degree, is perhaps true of the edge of the foot. In active creeping the anterior edge of the proboscis is kept in close contact with the substratum, as shown in figure 14; this organ undergoes 'spontaneous' local contractive movements, depending for their execution on the pressure of fluids contained in its interior spaces (Heath, '05 b).

b. The osmotic reactivity of Chiton's soft surfaces is important in connection with the question as to whether sugars are successful as activating agents for this animal. Sea-water of 36.5 per mille salinity (5/8 M) has at 27°C. an osmotic pressure of something more than 25 atmospheres, corresponding to a sucrose solution about 0.8 M. The limits within which various concentrations of sea-water do not stimulate were found to be 4/8 to 8/8 M (for the lips, the other regions being less sensitive); 1/2 sucrose in 5/16 M sea-water gave fair responses from all

parts, although 1 M sucrose in rain-water did not, except from the ctenidia. This total concentration ( $< 6/8$  M) is well within the 'osmotic limit,' and indicates that sucrose may be mildly efficient in stimulation. The behavior of maltose also shows faint indications (at 1 M in rain-water) of some stimulating capacity. According to Brooks ('16), sucrose penetrates (plant) protoplasm quite readily, and affects permeability after the manner of a monovalent kation, although not with special rapidity. The fact that in *Holothuria* (Crozier, '15 b) no evidence was obtained that sucrose could stimulate the integument—although maltose and glycerin apparently did, while in the experiments of Olmsted ('17 b) and of Hecht ('18) no sucrose effect was detected apart from that exerted through the osmotic pressure of its solutions—shows that some such factor as the

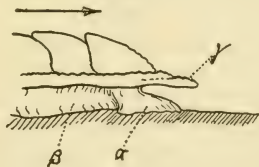


Fig. 14 Showing the manner in which the proboscis is kept in contact with the substratum during creeping. Anterior end, seen from the side:  $\alpha$ , proboscis margin ('palp');  $\beta$ , foot;  $\gamma$ , girdle. Arrow shows direction of creeping.

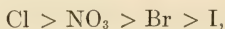
time of exposure to the sugar solution (or the concentration of accompanying salts?) may be important in determining whether or not activation occurs. Sensitivity to sugars, even if present, is however, undoubtedly very low on the general soft surface of chiton. With *Chromodoris zebra* we have found no stimulation induced by maltose or sucrose 1 M in rain-water, applied to various parts of the animal's surface in small volumes from a pipette, the animal being in sea-water. Similar tests with *Balanoglossus* (Crozier—unpublished experiments) resulted in no detection of activation by 1 M solutions of sucrose or of maltose.

c. Independently of their osmotic sensitivity, the soft surfaces of Chiton are also open to activation by dissolved electrolytes, which are much more powerful as excitants than are non-elec-

trolytes of the sugar type. The order of kation stimulating efficiency for the alkali chlorides,



is that found in the sensory activation of *Balanoglossus* (Crozier, '15 a), *Ascidia* (Hecht, '18), *Chromodoris* (Arey), and other marine animals, and is in accord with the general order of action of these ions upon various protoplasmic processes. The anion order,



does not agree with that determined by Hecht ('18) for *Ascidia*; but the methods of experimentation were in the two cases quite different, in the tests with *Ascidia* the method of limiting effective dilutions being employed and the salts being dissolved in sea-water. For *Synaptula* (Olmsted, '17 b), the order  $Cl < Br < I$ , as in *Ascidia*, was found, by the same general method. Each of these anion series has its counterpart in other salt actions (Höber, '14), but why they should be reciprocal is not altogether clear, particularly since the kation orders obtained by these respectively different methods are in agreement. In *Chiton*, general chemical excitation is primarily an affair of the kation.

The minimal concentrations of different electrolytes which are effective in the excitation of different regions of *Chiton* appear to be as follows (in sea-water solutions):

The three organic acids we employed stimulated according to the following order of efficiency:

Malic > lactic > acetic (M/100 in sea-water). This order indicates that for *Chiton* lactic acid is less efficient as a sensory excitant than malic, which is not the sequence shown (Crozier, '16 a) by the earthworm's reactions, nor in the penetration of tissues by these acids, but does correspond with the respective magnitudes of acid strength. Since the solutions were made up in sea-water, much emphasis cannot be placed on this point.

The very general nature of *Chiton*'s sensitivity, involving excitation by a great variety of materials in solution, adheres nevertheless to the rules already available in the activation of

numerous protoplasmic processes. The effects of salts, acids, alkalis, anaesthetics, and other substances in evoking reactions are best understood upon the assumption that cells of the external epithelium are acted upon by these substances after the manner of cells in general. Thus, with anaesthetics: chloretone (M/200) gave well-defined responses, whereas urethane (M/200) did not; the anaesthetic effect of these materials follows the same order (Crozier, '16 a). Some form of union with the surface of the epithelial cells is undoubtedly involved in the process of excitation, but these experiments cannot be understood on the assumption that excitation is determined by an increase in cell permeability. The decreased permeability produced by bivalent cations (e.g., Ca) is a specific function of the cation; nevertheless,

TABLE 5

*The minimal concentrations of various substances effective in the stimulation of Chiton*

REGION	KCl	HCl	KOH	Picric acid
Lips.....	N/160	<N/500	N/500	<M/1500
Ctenidia.....	N/50	<N/500	<N/500	M/1200
Edge of foot.....	N/100	N/450	N/400	M/1500
Sole of foot.....	N/32		N/400	M/1000±
Girdle.....	N/16	(>N/10)	(>N/10)	M/600±

while  $\text{CaCl}_2$  apparently does not serve as a sensory activator under the conditions of these tests, good reactions are elicited by  $\text{Ca}(\text{NO}_3)_2$  (compare also the case in *Balanoglossus*, Crozier, '15 a). There is no specific parallelism between efficiency in sensory activation and permeability-increasing properties. (Compare, for acids and alkalis, Crozier, '18 a.)

### 3. The chemoreceptors

a. The ventral parts of Chiton exhibit a chemical sensitivity which is essentially similar to that found for the general integument of other marine invertebrates (table 6). The limiting dilutions of various substances effective in activation of various parts of the animal (table 6) follow an order which is, for each



tissue, consistent among different substances. This suggests that—since the ‘thickness,’ ‘toughness,’ or density of these parts is in the same general order (lips > edge of foot > ctenidia > sole of foot > girdle), with the exception, perhaps, of the ctenidia—we are in reality dealing with a generalized form of sensitivity, the effectiveness in arousing reactions depending upon, 1) the ease with which the surface of the cells locally concerned may be actively penetrated by the excitant, and, 2) upon the relative richness with which these parts are respectively

TABLE 6

*Minimal concentrations of various substances effective in the sensory activation of various animals*

ANIMAL	HCl	NaOH OR KOH	NaCl	KCl	QUININE	PICRIC ACID	AUTHORITY
Man.....	M/1000	M/400	M/50		M/25,000		Parker ('12)
Ameiurus....	N/20	N/100	N/50		M/150		Parker ('12)
Amphioxus...	N/500					M/1,250	Parker ('08)
Ascidia <sup>1</sup> .....	N/625	N/100		N/4	M/2,500		Hecht ('18)
Balanoglossus.....	N/500	N/400		N/200	M/1,000		Crozier (unpublished)
Chiton.....	N/500	N/500		N/160		M/1,500	
Chromodoris.	N/700	N/200		N/10		M/10,000	Crozier and Arey (unpublished)
Synaptula....	N/600	N/200	N/4	N/40	M/10,000		Olmsted ('17 b)
Holothuria...	N/500	N/500		N/500			Crozier ('15 b)

<sup>1</sup> “Uncorrected” concentrations; Hecht’s paper ('18).

innervated. In a previous chapter we have shown that the tactile reactivity of these regions of chiton’s surface follows approximately the order:

head (palp, lips), ctenidia > edge of foot > girdle > sole of foot.

There is, thus, a distinct inconsistency in the relative sensitivity of these parts to touch and to chemical excitation, which shows that differences in the method of excitation undoubtedly exist

and that probably the relative richness of general sensory innervation is not the sole factor determining the chemical sensitivity of any one region. Further evidence for the distinctness of the chemoreceptors will be considered in the following section.

Through the comparison of the effectiveness in stimulation for different materials in Chiton and in other animals (table 6), it will be seen that the integumentary sensitivity of invertebrates corresponds in its general features (limiting effective dilutions) with that of taste in man rather than with the common chemical sense; there are, however, noteworthy differences from the physiology of taste excitation (Parker and Metcalf, '07; Crozier, '15 b). The fact that the isolated substances considered are, as such, foreign to the daily experience of Chiton, has nothing to do with the information they give concerning the process of excitation. That we are not concerned with general 'pain' reactions in the Chiton experiments can be shown in this way: The ventral surface of the girdle of Chiton, although relatively the most insensitive region to chemical excitation, is nevertheless decidedly reactive to touch. This region is excitable by pure anaesthetics, saturated sea-water solutions of essential oils, rain-water, by 10/8 M sea-water, and by 5 M glycerin, but is inexcitable (under the conditions of our experiments) by alkaline chlorides (other than KCl at 5/8 M concentration in rain-water), by KCl (in sea-water) more dilute than M/16, by HCl or KOH more dilute than M/10, by picric acid more dilute than M/700, or by ethyl alcohol even in 10 M concentration. Hence it would appear that only under conditions of an excessively heterologous quality may the ventral surface of the girdle be excited by these chemical agents, under such conditions in fact that 'pain,' tactile, or any other form of sense organ might be activated.

This result adds to the conviction that the general chemical sensitivity of Chiton's soft tissues is distinct from any form of tactile irritability, and is not consistent with the view that here—as there may be in *Balanoglossus* (Crozier, '15 a), or in *Synaptula* (Olmsted, '17 b)—there are 'generalized sense organs' (Nagel). Some indications are afforded that a distinct general chemical sense is adequately represented in Chiton. This con-

clusion may be tested through the attempted physiological isolation of chemical and tactile irritability. Such tests are, of course, open to several sources of serious error; the most critical results should be given by cases in which sensory exhaustion to chemical stimulation did not interfere with tactile irritability (Parker, '08, p. 440). A result of this kind is free from the objection that sensory fatigue may result in heightening the threshold to the more delicately acting forms of activation. Such a result is readily obtainable with *Chiton*: the ctenidia cease to respond to chemical activation by  $5/8$  N NaCl after about ten trials at brief intervals; they continue, however, to respond to touch.

This finding strengthens the opinion already derived from the distributional study of tactile and chemical activation in chiton. The fact that the reactions induced by these modes of activation are in some cases qualitatively identical, involving similar muscular contractions, is no obstacle to this view.

b. In the buccal cavity of chitons cup-shaped organs, with a suggested 'gustatory function,' have been described, as well as numerous nerve terminals in the subradular organ. We have nothing on this subject to add to Heath's ('03) observations, which we can confirm; these observations showed that positive food-taking responses are initiated by the excitation of the external surface of the mouth region with the materials upon which chitons feed. We have seen that mouth movements are initiated by chemical activation. It will be of interest to discover to what class of substances chitons react by food-taking responses.

Some writers speak of an osphradium situated at the base of each ctenidium in the chitons (Burne, '96). Pelseneer ('99, p. 13) has described and figured the two erectile ridges on the inner side of the posterior inner ventral border of the girdle (the "lateral lappets of the mantle fold" of Plate, '97, pl. 2, fig. 15, *llp.*), immediately caudad of the gills (figs. 7 and 8); he considers these structures to be homologous with the osphradia of other mollusks. In some species these protuberances assume the shape of well-defined papillae. They are situated on the distal face of the pallial nerve cords, which supply them with a rich

innervation, and constitute, in Pelseneer's opinion, special sensory regions, each of them being protected by the ventral face of the papilla, which is sometimes spiculose. We have described how the respiratory water current impinges on the dorsal face of each papilla. The conditions are therefore favorable for the situation of an organ "testing the quality of the water." The papilla may be significant for egg-laying responses (i.e., in the reception of a stimulus provided by the spermatid fluids), as it seems more prominent in the females. This remains to be tested. Copeland ('18) has brought forward good evidence that the osphradium of carnivorous snails is concerned in the reception of chemical excitation by dilute solutions of materials emanating from food; his further contention, that this organ is therefore an 'olfactory organ,' because the exciting agent is very dilute, is an unnecessary metaphor (cf. Arey, '18 b).

#### VIII. THE NERVOUS SYSTEM AND SENSE ORGANS OF CHITON

The main features of the foregoing account may now be briefly summarized. This report makes no claim to completeness; it does lay a solid foundation for further investigation in at least two directions: 1) the phenomena of seeming adaptation in the ethology of chiton, and, 2) the physiology of certain types of irritability. The sensory conditions are here unexpectedly complex. The major pathways of nervous transmission are, by contrast, unusually clear and well defined. The manner in which sensory capacities and modes of reaction are involved in the complex determination of natural behavior can be followed in great detail.

a. Tactile receptors are absent from the shell surfaces. The 'scales' and 'hairs' upon the girdle are important tactile organs. The ctenidia are also sensitive to touch, as are the proboscis, the foot, and the ventral surface of the girdle. The foot is positively thigmotactic to large surfaces, but retracts locally when stimulated by a small surface.

The tegmental aesthetes are photosensitive; they are activated by light of constant intensity and by sudden decrease in light

intensity, not by an increase. The dorsal surface of the girdle (scales) is also sensitive to light—characteristically to a decrease of light intensity, also to the constant intensity of light, and to a sudden increase in light intensity, provided this intensity be great. The soft ventral surfaces are sensitive to light. The periphery of the girdle is the ventral part most sensitive to shading.

The superficial soft tissues of Chiton are open to chemical activation, to stimulation by abnormal osmotic pressures, and by 'irritants.'

Evidence has been secured, through the study especially of the topographic distribution of the various types of excitability, that tactile receptors, photic receptors, and chemoreceptors are physiologically distinct. There is no clear evidence of sensitivity to heat; that to cold is less doubtful.

There is a pronounced tendency for the animal to come to rest in positions avoiding uneven tensions in the musculature. This is responsible for the precise negative geotropism exhibited by Chiton. This mollusk is not sensitive to vibratory mechanical disturbances.

b. This brings us to the consideration of one of the most unsettled problems in sensory physiology: In what manner is differential irritability determined? The immediate receptors of excitation in metazoans above the sponges are cells which function primarily as detectors and transmitters of disturbances in the energies of the environment. In a general way it is true that all forms of protoplasm are capable of being changed (activated) by light, heat, cold, pressure, chemical agents, and so forth. Considerations of this order, which hold also in certain cases upon the quantitative side (as in the action of chemical agents), have been responsible for the view that 'irritability' is a generalized property of living matter, best studied in uni- (or non-) cellular organisms. It does not seem probable that this conception can at present be of any great help; in spirit it is deductive, whereas the manifestations of irritability (e.g., in the diversified taste receptors of the human tongue) are manifold, specific, and must be investigated in a more purely inductive manner. Neverthe-



less, it is true that in those instances where photoreceptors, for example, may be isolated and distinguished, we frequently find that the sensory elements are removed from the external surface of the animal, protected from the action of environmental chemical disturbances and deforming contacts. From this standpoint one of the factors determining differential receptivity is to be found in the degree of anatomical isolation of the receptor; another, in the morphology of the sensory cell or organ, as in the development of distal projections. These factors of form and position undoubtedly facilitate the respective operation of different qualities of stimulation, and to that extent determine the functioning of differential irritability. But the problem is not in this way wholly solved. The evidence for 'generalized sense organs' is in some cases good, though perhaps not final. Even on the sole of the foot of *Chiton* physiological evidence of sensory separateness for photo-, tacto-, and chemo-reception is available. The skin of *Amphioxus* is fully as sensitive as that of other marine animals to touch and to chemical influences (Parker, '08), but is insensitive to light. The additional factor is probably found in the possession by certain receptor cells of special substances which enter into excitation reactions.

Even if in some cases it could be shown that the epithelial cells of an animal were open to sensory activation by a variety of means, it would not lead to the view that a 'universal' type of sense organ (Nagel) was that first developed in evolution, receptors of special kinds being by some obscure metaphysical process subsequently derived from it. In coelenterates, so far as we know (Parker, '17 a), tactile receptors and chemoreceptors are organically distinct.

c. The reactions of *Chiton* to local stimulation are of a character consistent with the known distribution of the central nervous system. At the sides of the body, those parts innervated by the pallial strands are conspicuously homolateral in their responses. The coördination of the pedal musculature for the production of locomotor waves depends upon a mechanism locally contained, and apparently upon the integrity of the pedal cross-connectives. The coördination of the gill movements on one side of the body

depends upon the intact condition of the pallial nerve strand on that side. The responses of isolated portions of an animal sectioned transversely are such as to show the absence of any strong centralization. This is in agreement with the known occurrence of ganglion cells throughout the whole length of the nerve strands. In *Chiton* nervous centralization is relatively at an incipient stage.

*d.* Alteration in the behavior of *Chiton* toward light with advancing age of the animal is the primary variable determining the exhibition of a very complex series of environmental interrelations. The young *Chiton* is photonegative, the old *Chiton* photopositive, to sunlight. Chitons of intermediate age are positive to weak light, negative to strong. Photoc orientation is direct, and is determined by the constant intensity of light, not by change of intensity. The progressive alteration in the sense of phototropism is determined by the erosive destruction of the photosensitive aesthetes, conditioning in older Chitons a lower specific stimulating power of the light. The erosion of the shell is in turn produced, in part, by, 1) normal growth effects; 2) the activity of organisms settling upon the shell plates.

The homochromic coloration of *Chiton* is determined by the nature of its algal food and by the organisms living upon its dorsal surface. The older chitons are relatively stationary; therefore specific local environmental influences have opportunity to affect the appearance of the chitons. The animals associate in groups, commonly of a certain average size and containing numbers of both sexes. Certain seemingly 'adaptive' consequences may reasonably be attributed to this mode of occurrence. A homochromically colored isopod is characteristically associated with *Chiton tuberculatus*.

These and other harmonious correlations, of which mention is made in the body of this report, follow automatically in the wake of the changing phototropism of *Chiton*. The animal's habits determine the environment in which it dwells. The precise and intricate bionomic correlations here briefly mentioned are an automatic consequence of its modes of reaction.

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# SENSORY REACTIONS OF CHROMODORIS ZEBRA<sup>1</sup>

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EIGHT FIGURES

## CONTENTS

I. Introductory.....	261
II. Mechanical excitation.....	267
1. Tactile stimulation.....	267
2. Righting; geotropism.....	275
3. Rheotropism.....	278
4. Nervous relations.....	282
III. Photic excitation.....	288
1. Light.....	288
2. Shading.....	292
3. Discussion.....	295
IV. Thermal excitation.....	296
V. Chemical excitation.....	301
VI. Summary.....	307
Literature cited.....	308

## I. INTRODUCTORY

With the objects of presenting data valuable for the comparative study of 'animal behavior' and of laying a foundation for the intelligent pursuit of certain inquiries in sensory physiology, this article aims to record and briefly to discuss some aspects of the sensory responses of the nudibranch *Chromodoris zebra* Heilprin.<sup>2</sup> In 1884 Bergh wrote that almost nothing was known

<sup>1</sup> Contributions from the Bermuda Biological Station for Research, no. 111, and from the Anatomical Laboratory of the Northwestern University Medical School, no. 68.

<sup>2</sup> Data upon which this paper is based were in part secured by L. B. A. during the summer of 1914. These observations have been extended and the paper written by W. J. C.

about the activities of the large exotic nudibranchs, and very little has since then been added to the subject. We shall deal with questions of natural history and behavior only in an incidental way, paying attention specifically to the manner in which *Chromodoris* responds when activated by various stimulating agents. It was desirable to undertake a study of this kind because *C. zebra* has already provided material, of a very exceptional character, for the treatment of some questions in which sensory phenomena are implicated (Crozier, '15 a, '16 a, '16 b, '17 d, '18 d). A knowledge of the sensory capacities and modes of response in *Chromodoris* affords, also, some information as to the comparative physiology of the nervous system in mollusks, about which, particularly in nudibranchs, very little is known.

*Chromodoris zebra* (fig. 1) is a large species very common at Bermuda,<sup>3</sup> with the form typical of the genus. An account of its morphology will be found in the papers of Smallwood ('10) and of Smallwood and Clark ('12). The body is elongated, especially in creeping, and measures up to 18 cm. in length. The animal as a whole is very soft and contractile, and becomes readily bent or twisted under appropriate conditions. Throughout the year individuals of a variety of sizes are to be had by dredging in depths down to 10 fathoms. From September to June great shoals of them, numbering thousands in all, crowd up at intervals into shallow water (Crozier, '17 b). They become notably concentrated in certain shallow mangrove creeks connected with Great Sound. The cycle of events which determines the shoreward flocking has not yet been fully established. Its coloration makes this nudibranch easy to distinguish upon the bottom (Crozier, '16 b), and the migratory movements of the species, owing to its lack of concealing behavior, may to some extent be followed in the field.

The animal moves with a smooth, even, gliding motion over rock surfaces or on the muddy bottom, the entire surface of the

<sup>3</sup> As with many other marine forms found at Bermuda, it is probable that the range of *C. zebra* is quite extensive, although so far it has been reported only from Bermuda (Heilprin, '89; Smallwood, '10). I am informed by Prof. W. H. Longley that a few individuals were obtained by his collectors, in seining on grass-flats at Tortugas. W. J. C.

foot being applied to the substratum. The whole body can, however, be supported by the use of a small part only of the foot; thus *C. zebra* has sometimes been observed to creep over the edge of a submerged rock, the body of the animal projecting horizontally beyond the edge, or its anterior part being even sharply elevated, until only a centimeter or so of the posterior region of the foot served as a hold-fast. *Chromodoris* can also swim attached to the surface film of quiet water, but has not been observed to do so in nature. (This behavior has been observed in another nudibranch, *Facelina goslingi*, at the time of its reproductive activity.)



Fig. 1 Outline figure of *Chromodoris zebra*; dorsolateral view from the right side (after Crozier, '18 d).

Especially in the mangrove creeks already spoken of, *C. zebra* is found in localities where eel-grass grows in great profusion. It creeps up the blades of eel-grass and is often found at the very tip. In connection with this habit it is important that the foot, although perfectly flat and without a trace of median division, when in contact with a flat surface, exhibits a most pronounced tendency to fold together, so that the lateral halves of the whole surface of the foot are in contact. This occurs whenever the whole or a part of the foot is removed from a substratum, and makes it possible for the nudibranch to climb the flat-bladed eel-grass. In *C. roseapecta* no such behavior of the foot is seen, and this species lives under stones along the shore, never coming into contact with eel-grass.

According to Vlès ('07), no pedal waves are observable upon the foot of *Æolis*, at least in the form of color differences, although the direction of the pedal wave was said by him to be direct, on the basis of the rippling movement seen at the edge of the foot during creeping. In *Doris*, also, the pedal wave was said by Vlès to be direct. In *Tectibranchs* the direction of the pedal wave, well defined in this case, is retrograde (Parker, '11, p. 156). The locomotion of *Chromodoris* is accomplished in the form of a smooth, planarian-like glide. No macroscopic waves have ever been seen on the foot, even when the animal was swimming attached to the surface film and therefore in a very favorable position for observation. Some unevenness of movement may usually be detected along the lateral margins of the foot, and, when fixed to the surface film, where locomotion is very slow, gentle 'billowing' movements can usually be detected. Hence it is possible, as Parker ('11) has suggested, that in this case the muscular activity of locomotion is not coördinated in wave form, but is arrhythmic. The lateral surfaces of the body and the surface of the foot are, however, richly ciliated. Much slime is laid down by the foot in creeping. The possibility, then, cannot be entirely excluded that progression is in part at least ciliary, especially since the cilia beat in the appropriate direction, namely from anterior to posterior. Small pieces cut from the mantle or from the foot continue to move for several days in a fixed direction about the bottom of a dish, owing to the beat of the cilia which they bear. According to Olmsted ('17), cilia are the means of locomotion in *Marginella*, *Haminea*, and *Bulla*. Local muscular activity, however, shares in locomotion, for the extreme lateral margins of the foot are the regions exerting suction when the foot serves as a hold-fast. This may be seen in the cup-like puckerings along the edge of the foot when 'swimming' along the surface film. These regions are not sticky, as if with slime, yet to a sufficiently large surface the outline of the foot, save at its anterior end, becomes so firmly attached that it is with difficulty pushed loose at any given point, although it can be loosened instantly when the animal begins to creep. Local movements of this muscular rim of the pedal



surface are visible during creeping. It is possible that the margin of the foot is alone concerned in active progression,<sup>4</sup> at least in so far as this is independent of cilia. With an individual attached to the vertical wall of a glass aquarium, the axis of the animal being horizontal, the lower edge of the foot sometimes becomes freed from contact with the glass, the creature then being suspended by the attachment of the upper margin of the foot; the median furrow is then plainly visible on the foot surface. On such a free lower pedal margin two or three distinct waves may be made out at one time. These waves, retrograde in direction (as in Tectibranchs), are confined to the outer margin of the foot. Similarly, in specimens resting in the angle made by the vertical wall of the aquarium with its bottom, the foot may be free anteriorly, being attached only at the posterior end. In these cases two or three waves were observed on each side of the foot at one time, the waves on the two sides having neither definitely 'opposite' nor 'alternate' relations, but appearing to be quite independent of each other. The fact that copious slime secretion occurs along the margin of the pedal surface in animals anaesthetized with  $MgSO_4$  or with chlore-tone enables a test to be made of the adhesive properties of this slime; it is not sufficiently sticky to cause the attachment of the foot to a glass rod, although in the unanaesthetized nudibranch such attachment is readily demonstrated. These several lines of evidence agree in pointing to the essentially muscular, non-ciliary, nature of the act of creeping in *Chromodoris*. It should be noted that the marginal pedal waves are retrograde, not direct, as Vlès states for *Doris*.

The direction of progression is always anterior. On a smooth surface the rate of creeping in active, large animals (at 27°C.) is about 1 cm. in five to seven seconds; when swimming on the surface film, about half this rate.

Although we are not concerned to give an exhaustive account of the effector systems of *Chromodoris*, mention may be made of

<sup>4</sup> In an unidentified species of *Leptoplana* I have observed locomotion essentially of this kind, obviously muscular, in which the outer edge of the body was the only part in contact with the substratum. W. J. C.

the fact that slime glands are important for the production of a slippery condition of the whole surface of the animal; and that repugnatorial glands, in part at least under nervous control (Crozier, '16 b; '17 a), are also involved in the creature's effector equipment. In addition to ciliary activity and several types of gland secretion, muscular movements of some variety are evidenced. *Chromodoris* has no hard supporting skeleton; its movements depend conspicuously upon the distribution, under muscular pressure, of the body fluids, and comprise: bending movements, twistings, contraction and extension of the whole body, and of its projecting outgrowths—tentacles, 'rhinophores,' and gills; protrusion and retraction of the proboscis, of the genital papilla, and of the oviduct; rhythmic contractions of the extended oviduct during egg laying, as well as local contractions of practically every part of the animal's surface.

*C. zebra* is functionally hermaphroditic, and reproduces at all times of the year (Smallwood, '10; Crozier, '17 b). The animals employed in this work were for the most part collected in Fairyland Creek, near the laboratory of the Bermuda Biological Station, where a practically unlimited supply of material was available during spring and early summer, when these experiments were chiefly performed. This nudibranch is easily maintained in aquaria (Crozier, '18 d), contrary to Smallwood's ('10) belief, but freshly collected individuals were almost always used. The largest specimens collected in late spring are the least viable; at a length of 16 to 18 cm. *Chromodoris zebra* undergoes natural death. It would be of interest to determine the growth rate of the animal, but this cannot as yet be attempted. A more detailed account of the natural history of *C. zebra* will be found in reports dealing with the phenomena of its breeding habits (Crozier, '17 d, '18 d) and of the coloration of the species (in course of publication; Crozier, '16 b).

## II. MECHANICAL EXCITATION

1. *Tactile stimulation*

The oral tentacles are very sensitive to touch, especially at the tip. When the tip alone is very lightly touched with a fine glass hair, it is contracted and slightly introverted. To slightly more intense stimulation, however, and always when touched at the side or at the base, the tentacle is introverted at the base after the fashion of a glove-finger. To unilateral stimulation of one tentacle, even to sharp and repeated touches, that tentacle alone responds. But after a tentacle has been completely, excessively, contracted, strong continued local mechanical stimulation of it (while remaining retracted) causes the opposite tentacle to be retracted. In this case the whole head region is more or less contracted, and it may be that the skin at the base of the retracted tentacle must be stimulated in order to result in a spreading of the response to the other one.

The 'rhinophore' on the same side with a stimulated tentacle usually contracts slightly, by a twitch of the muscles at its base, synchronously with the activated tentacle itself. If the stimulation is originally strong or if it is repeated, the opposite 'rhinophore' also responds, but usually to a less degree. Stimulation of a tentacle also involves response from the head region generally, causing it to retract; at the same time the buccal veil is drawn down so as to cover the whole mouth region, including the anterior edge of the foot. The anterior end of the body is under these circumstances contracted more strongly on the stimulated side, and after reextension the whole body is usually caused to bend in the opposite direction, away from the side originally stimulated. If the anterior part of the foot should not be in contact with the substratum, it also contracts, on the homolateral side, when a tentacle is touched. This general form of reaction is the common response when the nudibranch is stimulated anywhere with sufficient severity. Further evidence for the neuromuscular unity of the head region will be found in what follows. To a single light touch upon a tentacle, the general head response is very slight, but is nevertheless evident. The full head

reaction involves a deep insinking of the dorsum at the level of the 'eye spots.' This form of 'reflex' is seen also in other Dorids (*C. roseapicta*, *Lamellidoris*, etc.).

The tentacles do not easily become exhausted. After ten to fifteen successive applications of a glass rod, a tentacle is still reactive to light touch, although the resulting contraction is not so complete.

The 'rhinophore' of *Chromodoris* (Arey, '17, '18) is a somewhat complex structure. Its extreme distal tip is usually pale blue or white, the rest of the organ deep blue or purple. On either side of an anterior, median line, which is plain and smooth, the 'rhinophore' bears a series of twenty-eight projecting leaves. To a light touch at the extreme tip, a 'rhinophore' responds by partial retraction; the anterior, unmodified, median line is less sensitive; the posterior and lateral surfaces are the most sensitive. Even moderate intensities of activation cause a 'rhinophore' to be retracted within its collar, suddenly and completely, then reextended, more slowly. In animals of average size (8 to 12 cm. long) the 'explosive' type of response is the result of even light tactile stimulation. To a very delicate touch on the lateral or posterior face the retraction is only partial. The 'rhinophore' is itself contractile, longitudinal contraction occurring locally along its length when lightly touched, and it is pulled within its collar by the operation of basilar muscles within the 'rhinophoral' pocket. The retraction of a 'rhinophore' involves the subsequent sphincter-like closure of its basal collar.

A 'rhinophore' is not easily exhausted. When approximately the same spot on the side of the organ is touched fifty times at ten-second intervals, the amplitude of contraction decreases, but the 'rhinophore' is still reactive.

Stimulation of a 'rhinophore,' even repeated stimulation, does not influence the homolateral tentacle. The 'rhinophore' reaction is itself characteristically homolateral, as was seen particularly in the case of abnormal variates in which the 'rhinophores' were found naturally fused in varying degrees (Crozier, '17 e). A sharp tap administered to one 'rhinophore' results in the partial, less complete contraction of its mate, and also of the



gill crown. Less vigorous stimulation has no effect on gill contraction.

The gill plumes are individually sensitive, and react separately to slight stimulation. More violent activation (e.g., a sharp tap or gentle pinching) of a single plume spreads through the other plumes, according to its intensity. A single plume presents a smooth, narrow, distally tapering outer edge, a similar inner edge, and running between them two broad blade-like faces from which jut out the thin gill plates. Tactile excitation of the outer or of the inner faces leads to similar reactions of about equal magnitudes. The gill-bearing faces of the plume are less sensitive; frequently, an individual gill may be bent back and forth without eliciting a response. Presumably this occurs naturally in tidal currents, and during the movements of the gill crown as a whole.

To a light touch, the common form of response is a local constriction of the plume, usually not equal on the two sides, accompanied by local longitudinal contraction, so that a slight swaying movement of the plume results. The plume as a whole may or may not be pulled down at its base. To stronger stimulation the characteristic response involves the following events: local constriction, spreading distally from the point of activation, leading to the collapse and 'shriveling' of the plume distal to the point of activation; this is succeeded by the retraction of the plume through the traction of muscles not intrinsic to the plume itself, but situated in the basal tissue of the gill crown. Still stronger activation leads to longitudinal contraction of the gill plume, both distally and proximally to the site of touching. The reaction of the plume distal to the point of activation is nicely demonstrated by plumes which have acquired a branching or dichotomously divided form (Crozier, '17 e). If one of the branches of such a plume be touched on the side, this branch alone, and only distal to the stimulated point, contracts, unless the stimulation be too strong.

The type of polarity evidenced in the reactions of a gill plume is curiously akin to that seen in the responses of an actinian tentacle under similar conditions of local activation (Rand, '09,



'15; Parker, '17)—with this important difference: in the actinian tentacle it is the part proximal to the point of activation which contracts. The polarization of the gill plume, which is a neuromuscular matter since it disappears under magnesium sulphate anaesthesia, is further seen in the fact that the distal tip of a plume, when touched, gives rise to only a slight longitudinal contraction in the immediate region of the tip, although the basal contraction may lead to the retraction of the plume as a whole. Bionomically, the significance of the difference between the neuromuscular polarizations within the actinian tentacle, on the one hand, and the gill plume of *Chromodoris* on the other, lies in the fact that the actinian tentacle carries food to the animal's lips, hence the part between the disk and the point of excitation is shortened; whereas the mode of retraction of the gill plume probably saves it somewhat from being bitten by fishes. The gill plumes are bitten at by fishes, and there is evidence to show that some of the structural variations which they present (Smallwood, '10; Crozier, '17 e) originate as the result of injury.

The basal contraction of a plume spreads to other plumes in proportion to the intensity of the stimulus and to the nearness of its application to the base of the gill-plume; this is the reaction which is responsible for the retraction of the whole gill crown. Contraction at the base of a gill induces collapse of the whole plume.

Any desired degree of contraction of a single plume or of the whole set may be induced by grading the intensity of the tactile stimulus. Also by stimulating single plumes weakly and one at a time, as many plumes as may be desired can be caused to contract; e.g., all but one may be made to contract. Under slightly stronger activation, especially in the case of the more anterodorsal gill plumes, it can be demonstrated that the successive stimulation of two adjacent gill plumes is much more effective for the production of retraction of the whole gill crown than is the equivalent stimulation of any single plume. Thus, if two adjacent plumes are touched in quick succession or simultaneously the whole crown is retracted more or less completely;

whereas if either one of them is itself touched twice in this way, even if on the opposite faces, it alone reacts, though more vigorously than to a single touch. It is best to use large animals, with gill plumes widely extended, in testing this point, as otherwise the plumes may stimulate each other through mutual contact, a single slight stimulation then sometimes inducing relatively complete gill retraction. A single gill plume will react as many as twenty-five times in succession, when repeatedly touched at its tip, without mechanically involving another plume and without leading to contraction of the gill crown.

It would seem that the contraction of the whole gill crown when the gills are touched is a secondary phenomenon, depending upon the extent of the disturbance produced in the basal tissue as the result of the individual gill plume activation. Two gill plumes separated by two or three intervening members of the series do not, when touched in succession, lead to retraction of the whole gill crown.

The base of the branchial apparatus is also sensitive to touch. Stimulation of the brim of the anus, within the circlet of plumes, causes retraction of the gill plumes immediately adjacent to the stimulated site. To stronger stimulation of the anal brim, more and more of the plumes become involved in contraction. Activation of the anal brim is more efficacious in causing retraction of the gill plumes than is stimulation of the plumes themselves. The surface of the branchial organ outside the base of the gill plumes is very sensitive to touch; a slight stimulation induces complete retraction of the plumes.

It is difficult to fatigue the gill reaction. If the plumes are forced, through adequate stimulation, repeatedly to contract completely within the branchial collar, and the time required for subsequent expansion is noted, it is found that the time first shortens, then lengthens, as in this example (times in seconds): 85, 65, 45, 30, 23, 17, 15, 17, 23, 22, 23, 26, 30, 36, 34, 50, 55. The relative rate of exhaustion of the phases of the gill response is seen in the following experiment:

The anal brim was stimulated by touching it with a glass rod. After twenty-four applications, at successive intervals, the gill plumes still responded by contraction; although after the first twelve responses it demanded longer and harder stimulation to accomplish complete retraction of the gill crown.

In another animal the same form of stimulation was used, but the stimuli were supplied in groups of three successive touches. The individual touches were throughout of about the same force.

1st application of three touches—complete retraction within collar.

2nd application of three touches—incomplete retraction; not drawn within collar.

3rd application of three touches—about half the 'normal' response.

4th application of three touches—slight contraction only of the gills.

5th application of three touches—gills moved, but did not contract longitudinally.

6th application of three touches—gills moved slightly.

The integument of the head region is very reactive to touch. The anterior edge of the mantle fold (buccal veil) seems to act as the chief or immediately receptive part when the nudibranch during creeping meets obstacles raised above the general level of the substratum (fig. 2). A single stimulation of this part has a slight effect on the tentacles; repeated light touches cause first the homolateral tentacle, then the opposite one, to be fully retracted—or, if the median region of the mantle edge be touched, both simultaneously.

The reaction of the 'rhinophores' when the buccal veil is touched is very marked. It tends to be homolateral, as in the case of the tentacles, but is much more pronounced. The 'rhinophores' react as fully and as quickly as when they themselves are directly touched, although in the latter case the response is less easily fatigued. In some individuals this response of the 'rhinophores' is elicited by tactile activation of the dorsal integument as far back as the anterior level of the 'eye-spots'; in others, only as far back as the level of the 'rhinophores' themselves. In the region behind this level the effect on the 'rhinophore' becomes suddenly much weaker, and a response from the gill crown comes in, increasing in amplitude as places nearer the branchial collar are touched.

The dorsum of *Chromodoris* is soft, flexible, and very easily stretched and distorted. Therefore the delimitation of the re-

ceptive field for the 'rhinophore' reaction is best established with the aid of mild faradic stimulation. The electrodes can be placed in position without inducing local response and without leading to the reaction of distant parts, owing to mechanical deformation of the body wall. The receptive field of the 'rhinophore' reaction, made out in this way, agrees precisely with that already given. It is much more clearly defined than in the case of the gill response, as we have indicated above. Stimulation of a 'rhinophore' does not ordinarily lead to a reaction from the gill plumes, unless the 'rhinophore' be sharply struck or pinched.

The 'rhinophoral' collar reacts by constriction, sphincter-wise, when touched lightly, but first of all the 'rhinophore' is retracted. To a very light touch the stimulated part of the edge



Fig. 2 Outline lateral view of *C. zebra* (anterior end) during active creeping, showing the manner in which the lips, tentacles, anterior part of the foot, and the buccal veil are related to the substratum.

of the collar contracts locally without inducing the sphincter-like constriction and without leading to movement of the 'rhinophore.' When the 'rhinophore' is retracted as the result of being itself stimulated, the collar contracts over it.

The branchial collar behaves in a precisely similar way.

The projecting mantle-margin, if touched dorsally or ventrally, is locally depressed ventralward. This is also true of the caudal veil which carries on its ventral side the conspicuous mantle glands (Crozier, '17 a). When the animal is generally disturbed by being handled, or is from any other cause much contracted, the margin of the mantle along the sides of the body is thrown into prominence laterally, owing to the forcing of fluid into its internal spaces; whereas the caudal veil continues to be bent ventralward, unless the point of application of the stimulating



agent be on the caudal extremity of the foot or on the dorsal surface of the caudal veil itself (Crozier, '17 a). If strongly stimulated at one point on its dorsal surface, the projecting mantle margin is turned sharply dorsalward. Stimulation of the caudal veil leads to pronounced local contraction of the body musculature at that level, accompanied by contraction of the projecting 'tail' of the foot (analogous to the 'head response' at the anterior end of the animal). The peculiarities in the behavior of the caudal veil are related to the functioning of the large mantle glands, of which mention has already been made.

The peripheral edge of the foot is not very reactive to touch, excepting at its truncated anterior end. There, especially at the faintly projecting corners, corresponding to the place of origin of the 'foot tentacles' in other nudibranchs, it is very sensitive, a single touch causing head and tentacles to retract. Here again the response tends strongly to be homolateral. Stimulation of the lateral edge of the foot causes the foot to fold together lengthwise. This is also true of the sole of the foot (e.g., of an individual swimming at the surface film); a very distinct line, about 1 mm. broad, which owes its appearance to the vertical, dorsalward contraction of the foot muscles, makes itself apparent even when the foot may not fold together. Touching the margin of the 'tail' of the foot induces deep local puckering, and that part of the foot itself is pulled forward and upward.

The pharynx, when extended, is found very sensitive to touch. The lips of the fully protruded proboscis are relatively insensitive, but the very faintest touch upon its lateral wall leads to violent retraction of the whole head region; the pharynx is itself also momentarily introverted. Here, again, the reaction tends to be homolateral, as in all the responses of the head region. The oral area in animals with retracted proboscis is not so sensitive to touch as are the tentacles. In normal creeping it usually happens (fig. 2) that the proboscis is partially everted, so that a portion of its surface, exceedingly sensitive to touch, is brought into immediate relations with the surface over which the animal crawls. One-sided stimulation of the pharynx causes the homolateral 'rhinophore' to retract; stimulation of a



'rhinophore' does not affect the pharynx, unless it be repeated several times.

The genital papilla and the mouth of the oviduct, when everted, react locally to light touch, always contracting away from the point touched. They induce no general reactions of the whole body.

Chromodoris is relatively insensitive to vibrational stimuli. Continued tapping of the wall of a thin glass dish containing the nudibranchs may cause near-by resting individuals to begin to move, owing apparently to tactile irritation of the foot. No effect whatever is produced on creeping individuals, and no reactions are given under any circumstances by either 'rhinophores' or gill plumes. In nature the gills, 'rhinophores,' and mantle edge are moved about by tidal currents, and the body is by the same means caused to sway from side to side, without leading to noteworthy response, save in the case of the 'rhinophores'

## 2. *Righting: geotropism*

When the foot is removed from contact with a substrate, Chromodoris contracts to one-third or one-half its normal length, then subsequently becomes extended. The foot folds together longitudinally. The head end, after the preliminary re-extension of the body, is twisted on the long axis until the anterior part of the foot can be attached. The body is quite flexible, and in righting it may be twisted  $180^\circ$  or even  $270^\circ$  about its long axis. The foot is attached progressively, beginning at the anterior end; nevertheless, as already described, when once attached the posterior end is so well fixed to the substrate that the animal may be fully supported by this end alone.

The process of righting occupies about one minute, ten to twenty seconds of this time being taken up with the twisting of the body in the effort to secure contact by means of the anterior part of the foot. The anterior edge of the foot is the only part which becomes spontaneously attached in this way. Observations on many animals in the field, as well as in the laboratory, have shown that there is no pronounced tendency for *C. zebra*

to maintain an upright position of the body, with the foot ventral. The righting behavior is probably due merely to the stereotropism of the foot, especially at its anterior extremity. A *Chromodoris* placed on its back will become attached to a glass plate appropriately held in contact with the foot, even though the body remain upside down. This is best tested in *C. roseapicta*, where the foot does not tend to become folded together. The surface of the foot must be in contact with something. When removed from a substratum the foot folds together longitudinally so that the lateral halves of its surface are in mutual contact. The origin of the twisting movements is probably found in the mechanical excitations of the skin induced by placing the nudibranch on its side or back; the anterior edge of the foot also exhibits writhing movements when the animal is so stimulated, but upon getting into contact with a solid surface it reacts positively, by attachment and slime secretion, and righting is begun.

Repeated tests have been made to discover good evidence of geotropic orientation in *Chromodoris*, but without a decisive result being always obtained. Many individuals, in the light or in the dark, creep upward to the water's edge in an aquarium; but they also move downward, horizontally, or in any intermediate direction with perfect freedom. When situated on a glass plate which was tilted in various directions, they continued creeping 'as they were,' and could not be made to alter at the experimenter's will the direction of their creeping. These experiments were made at temperatures of 17° to 27°C. There seemed a somewhat more pronounced tendency to upward movement at 17° than at 25° to 27°, but the difference was not clear-cut and is perhaps fictitious.

If *C. zebra* possesses statoliths (otoconia) similar to those known in other nudibranchs, and perhaps of general occurrence in the group, they are not conspicuously involved in determining the direction of the animal's movements in the laboratory, nor the posture of the body in nature. It is of course conceivable that a vaguer type of geotropism is really functional, which might be difficult to detect in laboratory experiments. The

movements of the nudibranchs in nature are suggestive of this possibility (Crozier, '17 b, and section III of the present paper). For reasons subsequently discussed (section III), it seemed advisable to test the possibility of a relation between geotropism and the temperature.

The experiments already referred to were made at different seasons of the year, and the possibility was not lost sight of that the reproductive phase of a given individual might, through internal secretions or otherwise, be instrumental in determining, or in helping to determine, geotropic behavior. The natural movements of *C. zebra* are of very considerable complexity, and the following statements cannot be applied to the total analysis of these movements. These statements are based upon experience with many hundreds of *C. zebra* during the last five years.

During the winter months, at an average laboratory temperature (in the aquaria) of about 17°, *C. zebra* is notably geotropic, orienting upward and tending to remain at the water surface, especially when about to deposit eggs. This behavior is also notable in the field. After the egg mass is laid, the animal may wander downward again. At 27°, in summer, the same behavior is manifest, but less pronouncedly. Hence it is unlikely that the decided upward creeping in the first case is the result of oxygen-want.

The effect of oxygen-want, or of some associated condition, may be tested in two ways: 1) by observing the behavior of animals from which the branchial plumes have been removed or in which these organs are prevented from functioning, and, 2) by observing the behavior of *C. zebra* on a vertical surface in a jar closed above, containing no air space, but communicating with oxygenated water at its lower end. That the gills are respiratory organs is suggested not only by their blood circulation, but also by the fact that in sea-water of decreased alkalinity ( $p_H = 7.95-8.00$ ) the gill plumes become widely extended, the base of the gill crown being then inflated and protruded beyond the protecting branchial collar.

The result of such tests was as follows: Sexually ripe individuals tend to move upward, even though this be away from the

oxygen supply. 'Spent' individuals do not. When the branchial collar was sewed together so that the gill plumes could not be extruded, non-geotropic individuals did not tend to creep upward, but remained on the bottom.

The correlation of egg-deposition with negative orientation was very marked. For example, a group of fifteen nudibranchs had been in the laboratory for four months; during the last three and a half months of this time they deposited no eggs and remained for the most part at the bottom of their aquarium; suddenly, on the same morning, six pairs were formed, the animals crept up to the water edge, and deposited eggs, after which they wandered aimlessly.

If the temperature be gradually increased in a vessel in which *Chromodoris* is creeping upward under diffuse light, the anterior part of the foot becomes detached from the substratum when the temperature reaches 29° to 30°C., and if this temperature is maintained the animal creeps or falls to the bottom. This is probably an indirect effect of temperature upon geotropism. It is possible that the reproductive mass, enlarged when ripe, acts as a statolith; if this is correct, geotropic orientation may result: from, 1) a general increased irritability accompanying sexual ripeness, plus, 2) the mechanical stimulation of the loosely anchored internal organs; on a vertical surface, the animal would then turn away from the side against which these organs pressed. This would result in negative geotropism, as found. Together with positive phototropism (*vide infra*) and a negative reaction to high temperature, geotropic behavior might, then, be important for the determination of the vertical migrations of the species into shallow water at periods of breeding; it would nevertheless be incorrect to say that the animal "moves into shallow water for the purpose of breeding."

### 3. Rheotropism

In some of the situations where *C. zebra* abounds, as, for example, in Fairyland Creek, the nudibranchs are well exposed to the possibly directive influence of tidal currents of considerable

volume and velocity. The habitat of this animal is preeminently within the semienclosed lagoons or sounds at Bermuda, where tidal currents must frequently be encountered; it does not occur upon the reefs. It was important to discover the nature of the animal's rheotropism, if it should be found to be oriented by water currents. Since the usefulness of information upon this point lay in its application to the natural movements of the animal, the experimental work was done in the field. Laboratory tests, moreover, were found unsatisfactory because water currents of sufficient volume could not be employed conveniently.



Fig. 3 Chart of a portion of Fairyland Creek (F. C.), showing the situation (cross within a circle) for testing the behavior of *C. zebra* in tidal currents (see text). *A* is drawn to a scale of 6" to 1 mi. *B* is an enlarged sketch of the region within the rectangular area surrounded by dashes in *A*.

An appropriate situation was found at the western end, or mouth, of Fairyland Creek (fig. 3), in a locality where hundreds of the animals were living at the time the experiments were made, and involving, therefore, water currents normally encountered by the nudibranch.

At the period of the falling tide the currents in the location selected were as shown in figure 3. The observations were at first confined to sunny days. When low water or high water occurred at about midday—no water then (for a short period) flowed across the channel indicated—the sun was sufficiently



far south (in December) to cause the nudibranchs, not singly, but by the dozen, to migrate southward in this channel. During falling tide, with the current flowing northward, the nudibranch, moved with the current; but once out of the channel they tended to turn around so as to face the sun as soon as they were out of the current. In this way groups of fifty to sixty individuals were caused to collect just beyond the northern end of the little channel. For the study of rheotropic orientation a flat slab of rock was placed horizontally in this channel, and nudibranchs were placed up on it in various positions with reference to the current. In some cases the *Chromodoris* was allowed to become attached to the rock while surrounded by an inverted glass jar which temporarily protected it from the action of the current. So long as the current was of fair velocity, orientation was always precisely negative, the nudibranch moving with the current. Under these conditions, the whole body is swayed to one side or the other by the force of the current, the gill plumes are moved by it, the 'rhinophores' are bent backward or to one side, and the buccal veil of the mantle is irregularly distorted. The gill plumes and 'rhinophores,' in particular, are forcibly moved by a current too weak to noticeably affect the body as a whole in a grossly mechanical way, yet leading to precise orientation in the current. It was considered that some or all structures mechanically distorted by the water current might be responsible for the negative rheotropic orientation.

Experiments were begun with the 'rhinophores.' When exposed to mild water currents of sufficient volume, as in the natural channel already considered, the 'rhinophores' are forced backward (fig. 5). When the current is stronger, the position assumed is as shown in figure 4. The 'rhinophores' are easily removed by seizing with forceps and cutting close to the collar. In one experiment six nudibranchs from which both 'rhinophores' had been removed the previous day (they crept about in an entirely normal fashion, for removal of the 'rhinophores' has no seriously adverse effects) were found not to be oriented by a current in the natural channel, although a dozen or more individuals with intact 'rhinophores' oriented precisely. The

rhinophoreless individuals assumed a position like that in figure 4, and the gills and buccal veil were forcibly distorted to a maximal extent by the current, but no orientation took place. A group of fifteen nudibranchs was then prepared, from nine of which the right 'rhinophore,' and from six the left, was removed. When the current was allowed to impinge on the anterior end of the nudibranch, parallel to the long axis of the animal, in almost every case orientation was prompt, and the bending of the body took place in such a way that the side contracted was the one carrying the intact 'rhinophore.'

Experiments were also made with more localized currents. A stream of sea-water flowing through a tube of 6 mm. bore at a speed of 4 to 5 cm. per second was allowed to impinge horizontally

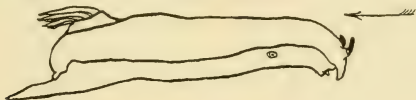


Fig. 4 Showing the posture of the body in a *Chromodoris* exposed to a tidal current (see text).

upon the anterior end of *C. zebra*. To this current normal individuals became promptly oriented, the process occupying three to five minutes (at 17°C.). Animals without 'rhinophores' were conspicuously slow and unsuccessful in orienting away from this current, although the buccal veil and the gills were moved by the current to an equal extent in both cases. The negative orientation to the current did occur in some cases, but only after half an hour or longer. The 'rhinophores' are easily distorted by currents and do not retract when moved in this way. A current of small cross section, affecting only the 'rhinophores' (fig. 5), causes the animal to bend toward the unstimulated side.

These results leave no doubt that to currents of adequate velocity the nudibranchs are negatively rheotropic and that the 'rhinophores' are the prime receptive organs for this kind of reaction.

#### 4. *Nervous relations*

1. There are several very characteristic features about the sensory responses of *Chromodoris*; these are of considerable general significance. Yet the apparent variability of these responses has made it necessary to study them very carefully and in many individuals.

To local excitation, not too intense, the response is local merely; to more vigorous stimulation, the response obtained involves more distant structures—at the anterior end, the general head contraction; at the posterior end, the caudal contraction;

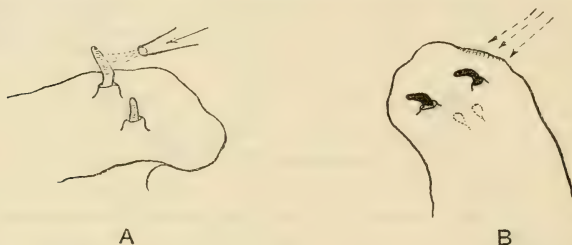


Fig. 5 Behavior of the 'rhinophores' in a water current; A showing the 'positive' reaction of the 'rhinophore' itself; B indicating the method of reaction to local current affecting directly only the 'rhinophores.'

likewise at the anterior end the 'reflex' involvement of other structures than the one stimulated proceeds upon a conspicuously homolateral plan. In the case of the tentacles and 'rhinophores,' moreover, stimulation of a tentacle easily induces contraction of the homolateral 'rhinophore,' whereas the reverse operation is exceedingly difficult.

The manner in which, at the head end, additional structures become concerned in reaction to the activation of distant parts, corresponds precisely to the distribution of the main anterior nerve trunks; and the character of the responses, particularly in the apparently non-reciprocal nature of conduction between tentacle or pharynx and 'rhinophore,' is strongly suggestive of true reflex action.

2. On the other hand, in the case of each of the projections from the body (tentacles, 'rhinophores,' particularly the branchial plumes, and perhaps the pharynx) the local reaction of each stimulated part has certain definite peculiarities, best studied in the gill plumes, but seemingly identical in all the parts enumerated. These peculiarities are: localized longitudinal contraction at the immediate side of activation; circular constriction and longitudinal contraction beyond (distal to) the level of activation; contraction at the base when the activation is sufficiently intense, in this case involving a spreading of the response to neighboring parts; a lesser reactivity when the tip of the organ is activated than when it is touched near its base; in the gill plumes, neuromuscular polarization such that the activation spreads distally from the point of excitation; slight fatiguability of the local reactions, whereas the heterolateral responses (e.g., in case of the 'rhinophores' and tentacles) are much more readily exhausted by repeated activation.

The local responses are exhibited in pieces of the mantle removed from the body. When the central nervous ganglia, supra- and subœsophageal, have been completely extirpated, stimulation of the head region near a 'rhinophoral' collar causes that 'rhinophore' to be retracted, the collar closing over it, as normally stimulation of a tentacle leads to its reaction, but does not involve retraction of the homolateral 'rhinophore.' Tactile excitation of a 'rhinophore' in a *Chromodoris* with the ganglia excised causes the 'rhinophore' to retract, after which it is slowly re-extended.

The phenomena of local response to faradic stimulation in the excised gill plumes are also substantially similar to those of the individual plume in the intact nudibranch. Within fifteen minutes after amputation a gill plume becomes relaxed, though, like the excised tentacle of an actinian (Parker, '17), it is not so fully extended as it may be when attached to the animal, because no fluid is being forced into it. The relaxed, isolated gill plume is fully as sensitive to touch as when forming part of the normal nudibranch, the peculiarities of its reactions are identi-

cal, although it is somewhat more quickly exhausted and only rarely responds at all to shading. The responses disappear under chloretone anaesthesia, but return again in sea-water. The neuromuscular polarization of the gill plume is therefore a local matter, conditioned by a self-contained nervous apparatus which conducts impulses more easily distalward than proximally.

These facts speak unmistakably for the presence of local peripheral conducting paths, having the characteristics of true nerve nets. Similar nerve nets have already been identified in *Octopus* (Hofmann, '07), and in *Aplysia* (Bethe, '03).

The body of *Chromodoris* may be laid open by a dorsal or a ventral incision, and the animal will live for a long time in sea-water. The nerves which originate from the 'cerebral' and sub-oesophageal ganglia and traverse the body cavity are readily employed for faradic stimulation experiments. The results of such tests confirm Bethe's ('03) description of the effects of nerve-trunk stimulation in *Aplysia*. Local responses, of no great magnitude, are induced; much more general effects are obtained, with the same stimulus intensity, when the integument is activated directly. These experiments incidentally afforded information relative to the old controversy as to whether the projecting marginal ridge is an epipodium (Herdman, '90; Herdman and Clubb, '92) or a mantle structure proper (Pelseneer, '94, p. 70). Pelseneer was undoubtedly correct, at least so far as our species is concerned, for the motor nerves to this region are pallial; not pedal.

3. The general result of these experiments is to suggest the probability that peripherally a true nerve net is concerned in local sensory responses, but that a reflex system involving central conducting paths is called into play by more intense activation. We are able to offer in addition physiological proof of a different kind that the peripheral conducting systems are nerve nets, and that the central paths of nervous transmission are part of a synaptic system, to which the term 'reflex' may properly be applied. This proof is based upon the assumption that the effect of strychnine affords a good test of synaptic transmission.



The following notes are derived from observations with eleven *Chromodoris* of medium size (10 to 14 cm. in length) into which 1 cc. of half-saturated strychnine hydrosulphate in sea-water had been injected. This quantity was found by other tests not to be fatal and to be the optimal concentration for our purpose. The injection was made into the region of the heart, on the dorsal surface. The behavior of each animal was studied individually, before injection, during the action of the strychnine, and after its effects had worn off. As a control, each individual was studied in comparison with an animal into which 1 cc. of sea-water had been injected. The latter operation had no detectable consequences of any kind. Tactile activation was mostly used. The results herein summarized are to be compared with those given in the first section (p. 267).

Following strychnine injection, the body remains for some minutes much contracted, its surface being 'wrinkled' and thrown into edematous blebs; the genital papilla is protruded, and the posterior mantle glands are made prominent, owing to the forcing of fluid into the spaces surrounding them. These effects appear under any conditions leading to pronounced general contraction of the body muscles. The gill collar, however, is strongly contracted in a peculiar way, its edge being rolled outward. The foot is folded together lengthwise and does not attach to the substratum. The gill plumes remain half contracted within the branchial collar. The plumes tend to exhibit more or less rhythmic contractions, followed by rapid but incomplete expansion; perhaps this is in some way mechanically induced by the beat of the heart, which distorts the neighboring dorsal integument. The reaction of the gill plumes to shading is not apparent.

After the lapse of half an hour to an hour in different individuals, the body is less strongly contracted, the gill plumes more fully extended. The reactions of the plumes to touch are curious and important at this point: to a single touch, a plume reacts precisely as in non-strychninized individuals; but when two successive touches are administered to adjacent plumes, the reaction is of unexpected violence (fig. 6). A reaction of this amplitude is obtainable in normal animals only by six or seven

repeated proddings of the gill crown, but relatively slight taps of adjacent plumes will produce this effect under strychnine. The 'rhinophores' are not retracted under these circumstances; whereas, if the 'rhinophores' themselves are touched, the gill plumes do contract.

In some individuals the 'rhinophores' were found to retract noticeably, but not completely, when a bit of graphite or the end of a glass rod or of an aluminum wire was brought near them (within 2 to 3 mm., but not touching). Presumably this represents a heightened tactile irritability such as that seen in some teleosts (Crozier, '18 c) after the removal of the eyes (i.e., when the central reflex interference of optic impulses has been re-

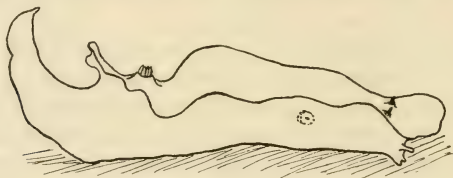


Fig. 6 Outline of *Chromodoris* to show gill-crown reaction under strychnine.

moved). This type of irritability is not apparent in the non-strychninized animal.

After one hour, the dorsum is still wrinkled, but the animal attempts to creep, usually falling over to one side after such an attempt has endured for two to three minutes. The main body is no longer forcibly contracted, but usually assumes a gentle spiral form about the long axis, the head pointing downward on one side, the 'tail' of the foot pointing upward and to the opposite side; the surface of the foot is for the most part longitudinally folded. Touching a 'rhinophore' causes both it and its mate to retract; sometimes the opposite 'rhinophore' contracts before the stimulated one, and usually the gill plumes contract also. The lightest touch applied to a tentacle causes the homolateral 'rhinophore' to be fully retracted. When a 'rhinophoral' collar is touched, it contracts, sphincter-wise, so quickly and so forcibly,

that the 'rhinophore' has difficulty in being itself retracted within its pocket; this behavior is never seen normally. If the anterior edge of the buccal veil is touched at one side, there results, as in the normal nudibranch, a homolateral 'rhinophore' retraction, and also a retraction of the gill crown, which is rarely seen except under strychnine.

After one and one-half to two hours, the pharynx invariably becomes extended; if touched at the side, an exceedingly violent homolateral head response is the result. If the lips be touched, however lightly, both 'rhinophores,' as well as the proboscis, are violently retracted. On reextension, the 'rhinophores' both retract when either one is touched, but the pharynx (extended) does not contract at all. Nor at any other time does 'rhinophore' activation induce retraction of the pharynx.

In all of these reactions, for example, when both 'rhinophores' retract as the result of one of them being touched, it is very difficult, if not impossible, to secure the double response for five to ten minutes subsequent to the reaction, although each one responds to local activation readily enough after a 1- or 2-minute refractory period; this is true of the double response independently of whether the 'rhinophore' first activated or the opposite one is the one subsequently stimulated. A precisely similar relation appears in the other responses studied.

This period is usually succeeded by one (two and one-half to three hours after injection) during which a light tactile stimulation of one 'rhinophore' causes both the opposite one and the pharynx (still extended) as well as the tentacles and gill plumes, to be retracted.

These effects of strychnine injection become obliterated after the lapse of three and one-half to four hours, under the conditions used in the experiments, and the animals return to an essentially normal state so far as their reactions are concerned. At no time is the response of the gill crown to shading (*vide infra*) in any way enhanced.

4. If these results are compared with those given for normal animals (section 1), it will be seen that strychnine has a pronounced effect upon those responses involving irreciprocal conduction,

and upon the reactions shown by experiments, with the central ganglia eliminated, to involve central transmission tracts. The progressive development of the strychnine effect, in point of time after injection, upon the several responses investigated; the pronounced refractory phase following each response involving the strychnine effect, and the enhancing nature of the effect itself, all point to the reflex nature of the nervous transmission concerned, since these effects are precisely those which strychnine exerts in decreasing synaptic resistance. On the other hand, the local responses, as seen particularly in the gill plumes, are not materially affected. Strychnine does not exert these effects upon de-ganglionated *Chromodoris*. Consequently we may assume, although we have not inquired as to the specific character of the strychnine effect, that peripherally, and in the outgrowths of the body wall (tentacles, pharynx, 'rhinophores,' gill plumes) there are local nerve-nets concerned with local responses, that these nets are characteristically polarized, and that they are dominated by the central nervous system of the nudibranch, the latter being essentially a synaptic system.

### III: PHOTIC EXCITATION

#### 1. *Light*

*Chromodoris* is sensitive to light. Most of the many individuals tested oriented directly toward a source of sunlight or of diffuse daylight. The tests were frequently made in a rectangular glass dish, enclosed in a covered dark chamber admitting light through a slot in the bottom of one end. Most of the individuals moved immediately toward the light aperture and remained for a long time pressed against this end of the aquarium. Some continued to creep around the side of the dish when they came into a shadow, but ultimately, on coming again into the light, oriented toward it. Orientation is direct, without trial movements, and the anterior end does not react to shading; neither does it respond to increase of light intensity as such. In the great number of individuals which we have handled at different times, no exceptions were ever found to the occurrence of



positive prototropism, although many individuals may for a time be inactive and may appear insensitive to light. This is notably true when two or more individuals are together in a dish, in which case, if they be ready for pairing, they may stimulate one another to conjugation. Even here, however, photic irritability is evidenced in one respect, for in the dark (in well aerated water) the gill crown is almost invariably contracted; upon illumination, even by the light of a match, the gills become extended; this occurs also during the progress of copulation in the dark.

The directive effect of light is manifest not only in horizontal creeping, but also when the illumination is from above. Sunlight reflected from out-of-doors was caught by a second mirror and reflected vertically downward into a vessel containing *Chromodoris*. Active locomotion ensued, and many animals on reaching the side of the jar climbed it until they met the water surface, continuing then along the water line; whereas, on creeping outside the path of the light beam, the nudibranchs tended to return to the bottom of the dish. In successive tests the animals could be forced to creep upward when illuminated, going downward again during intervals of shade.

The effect of illumination is distinctly a kinetic one. In a shaded dish the nudibranchs become quiet, but are set into activity at once if light be thrown on the dish. In spite of their positive phototropism, these nudibranchs tend under some conditions to collect in the shade. In a shallow dish well shaded on one-half, the dish being in a chamber admitting direct sunlight from above on one-half, there seemed to be a decided tendency for the *Chromodoris* to collect in the shade; whereas when diffuse light was used the light compartment was the one more frequented. This is explained by the fact that strong light induces greater activity, leading automatically to wandering movements, which become less pronounced in the shade, while with diffuse light the photopositive behavior is not interfered with by photokinetic effects. If originally placed in the dark compartment, the *Chromodoris* wanders into the light. Even with direct sunlight, falling vertically into the light compartment, no reaction other than increased speed of creeping is detected.



The eyes of *Chromodoris* are small and are situated beneath the skin. They are in close relation with the supraoesophageal ganglionic mass, being connected therewith by means of short slender nerves running to small optic ganglia (Smallwood and Clark, '12). Externally, the region of their location in the normally extended animal is indicated by two clear-blue areas immediately posterior to the 'rhinophores' (fig. 1). In nudibranchs found by previous test to be actively photopositive, these regions were cauterized with a hot needle. Subsequent experimentation showed that the photopositive behavior of these individuals was in no way affected by the operation. A scar-like formation was produced, accompanied by some local puckering, together with a deep dorsal constriction and insinking of the body at the level of the burn. The movements and general behavior of the animal are in no way altered.<sup>5</sup>

It is doubtful if operations of this type really interfere with the possible functioning of the eyes. Nevertheless, when tested with small areas of illumination, even when the light was very intense, the normal *Chromodoris* was found to be reactive at the anterior end, in the region of the eyes; the posterior end was non-reactive to sunlight concentrated with the aid of a lens of 30 cm. focal length when the heat rays had been eliminated. We are not in a position to decide as to whether the eyes are photosensitive (for they are not easily approached for excision tests), nor whether there are anterior integumentary receptors independent of the eyes. The 'rhinophores' are not sensitive to light.

The gill plumes, however,—more or less completely retracted in the dark, as already described,—become fully expanded when the region of the branchial collar alone is illuminated. They do not become extended, it would appear, when only the anterior end of the animal is illuminated. The latter test is difficult to make because anterior illumination induces active creeping.

<sup>5</sup> The region of the skin bearing the eye spots has also been removed in a number of cases, exposing the body cavity. Animals so prepared are photopositive; they live for a week or more and seem in no way greatly incommoded by the operation. Through such a window in the skin it was attempted to stimulate the eyes directly, with a small spot-light. It seemed that the region of the eyes was sensitive to light, but the experiment should be repeated.

There is, at any rate, an instructive correlation in these responses: illumination induces creeping, and also induces extension of the branchial apparatus; it is to be presumed that increased oxidation necessary for locomotion is in this way assisted. The photopositive behavior of *Chromodoris* is not accompanied by any reactions to changes of light intensity. It is an example of phototropism in the strict sense, in which trial movements do not appear.

Long-continued observation of *Chromodoris* in the field has shown that the positive phototropism of this nudibranch is of great bionomic importance (Crozier, '16 b, '18 c), a preponderating element in natural behavior. On days when the sky is overcast, relatively few of them are to be found. The brighter the day, other things equal, the more of these nudibranchs one can collect in shallow areas. In suitable spots they can be observed to follow with precision the direction of the sinking sun, whether it leads up-hill or down, according to the nature of the bottom. In this photopositive behavior *C. zebra* agrees with some Red Sea chromodorids, as described by Crossland ('11), and differs sharply with the behavior of *C. roseapicta* at Bermuda and with tropical chromodorids in general as indicated by Eliot's ('04, '10) experience.

In any one locality where they abound, more of the nudibranchs are obtainable in mid-afternoon, on a sunny day, than in the morning. But this is true only during the cooler months of the year (October to May). During the summer, none, or a few only, are so obtainable in shallow water (e.g., in the mangrove creeks or on shallow grass flats), though search among the densely packed eelgrass usually shows that in June and in July a certain number are still there. If one collects at early morning, before sunrise, in Fairyland Creek, relatively large numbers of *C. zebra* can be had in June and in early July. As the sun rises, the nudibranchs creep downward on the blades of eelgrass and turtlegrass, and by the time the sun has risen several hours, practically none are to be seen.

In view of the positive phototropism of *C. zebra*, consistently exhibited by individuals of every size, this phenomenon was very

puzzling—especially since it was found that nudibranchs obtained (in Fairyland Creek) before sunrise, and tested immediately with lateral light, were without exception photopositive. No reversal of the customary phototropism occurs, under these conditions, at the time of sunrise. Nor is *C. zebra* photonegative immediately after long exposure to the dark; but even if this should be true, it would not explain the natural behavior described.

Several possibilities were considered, among others the possible reversion of phototropism by rise in temperature. The temperature of the water in Fairyland Creek was 24° to 25°C. just before sunrise. The water is very shallow, and is rapidly heated by the sun's rays so that it quickly reaches a temperature of 27° to 28° as the sun rises. But the phototropism of *C. zebra* is not altered at any temperature between 17° and 31°; at the higher temperatures orientation is quicker, and still toward the light. Other possibilities are dealt with in a preceding section (p. 277).

The positive phototropism of *C. zebra* is not affected by prolonged starvation (four months; Crozier, '18 c), and is the same in sense with animals dredged at various depths down to 8 fathoms; nor does it vary with the reproductive condition of the animal.

## 2. *Shading*

The gill crown of *C. zebra* reacts to shading, after a detectable latent interval. No other portion of the animal's surface is sensitive in this respect. The gill plumes must themselves be shaded in order to produce a response. The reaction in question is in the form of an incomplete retraction of the gill crown, accompanied by longitudinal contraction of the individual plumes. The responses are exceedingly variable. The first reaction obtained from an animal which has for some time been undisturbed, in the light, is likely to be the most pronounced. This is not always true. Subsequent successive shadings commonly evoke a faint contraction of the plumes, the crown as a whole being little if at all retracted. This reaction is precisely similar to that which may be induced by tactile irritation of the gills, but the

time occupied by the contraction process is less in the case of a shading response (0.6 to 1.0 seconds) than in a tactile reaction involving about the same degree of muscular activity.

The duration of the shadow must be appreciable to have a detectable effect. The duration increases with the size of the animal. For nudibranchs 10 to 12 cm. long, an opaque body, such as one's hand, moving at about 50 to 60 cm. a second between the gills and the sun provides very nearly the threshold of activation. With the removal of the shading the gill plumes expand; they tend to remain contracted in the dark, provided the whole animal or its posterior end is shaded. If, while the plumes themselves are contracting as the result of shading, the light be suddenly or slowly increased again, the retraction of the gill crown is inhibited and protrusion begins.

It was previously shown that the total response of the gill crown involves two reactions: contraction of each gill plume and the retraction of the whole branchial apparatus. These are quite distinct things. The retraction and extension of the gill crown is brought about by muscles at its base. This is determined by the acting light intensity, owing apparently to the fact that bright light decreases the tonus of the muscles in this region, allowing fluid to accumulate there, under pressure from other portions of the body. The reflex contraction of basal muscles causes the crown to be retracted. The contraction of the individual plumes, however, is determined by shading as such; because, after the initial twitch or longitudinal contraction of the plumes they become relaxed even if the state of decreased light intensity continues. The plumes do not react to suddenly increased illumination. The degree of contraction of the gill plumes when shaded determines whether the whole crown shall be retracted or not. The relations here are very similar to those previously discussed under the head of the tactile activation of the plumes. If the self-contraction of the plumes be sufficiently violent, more or less complete contraction of the whole crown ensues; in this case the reextension of the gill crown commences within a few seconds, as under tactile activation, even though the shading remains constant and the crown may not be fully reextended. The



shading response is therefore to be sharply distinguished from gill-crown retraction; the former is a local matter, involving the plumes individually, through their local and probably non-synaptic nerve nets (since strychnine has no effect whatever in increasing the shading response); the latter is a reflex effect (compare strychnine experiments cited in section I), so far as contraction under shading is concerned, and when not secondarily involved, owing to the gill plume reactions, is governed solely by the constant intensity of the light (granted optimal conditions of oxygen supply).

The extent of the gill-plume reaction to shading is very variable in different individuals, and it has not been possible to control this variability. Sometimes every animal in a dish was found markedly sensitive, in other cases only one or two gave detectable reactions. Subsequent investigation showed that strands of slime connecting one individual with another occasionally caused one sensitive animal to stimulate others confined with it (Parker, '08, for a not dissimilar instance in the behavior of *Amphioxus*). Even when studied in individual aquaria, however, great variability was found. Sensitivity to shading was not enhanced by confinement in the dark or in the light even for lengthy periods.

Successive shadings at 30-second intervals elicit responses growing rapidly more feeble, commonly failing after the third or fourth. Here again, however, variability is very great; one individual gave twenty such successive reactions.

Sunlight, diffuse daylight, light from an incandescent bulb, were each efficient for the production of shading reactions. The visible region of wave lengths is concerned, since responses are obtained on shading through several thicknesses of glass. Tests with ray filters showed that the cutting off of light passing through a blue filter ( $\lambda$  523-450) produced good reactions, whereas that through a red filter ( $\lambda$  690-634) most often failed. Experiments with green and yellow filters gave no clear result. This agrees with results obtained in similar experiments, employing the same ray filters, with a barnacle (Crozier, '15 a, p. 273) and with *Chiton* (Arej and Crozier, '19).



Some other nudibranchs, as *Hermæa* and certain *Aeolids* (Garstang, '90, p. 423), and *Facelina goslingi*, are also reactive to shading. In *Facelina* the anterior end is the sensitive part. It responds to decreased light intensity by a quick retraction of the head, followed by its rapid reextension. The head end must itself be shaded in order to produce this effect. The response occupies 0.6 to 1.0 second, and is followed by a 0.5 to 1.0 minute refractory period during which no shading response can be elicited. It is very difficult to fatigue this reaction, probably owing to the long refractory period; after twelve to fifteen successive shadings and reactions there was no evidence of exhaustion.

### 3. Discussion

On the ground of their respective modes of distribution upon the surface of the animal, it would appear that tactile and photic irritability are served by quite distinct receptive mechanisms. Especially is this so in the case of shading. After complete exhaustion of the shading response, as well as during the brief refractory periods in which shading is non-effective, tactile irritability of the gill plumes seems not in any way impaired. Since precisely similar motor effects are concerned, we may conclude that exhaustion of the receptors for shading does not interfere with tactile irritability, and hence that the receptive mechanisms are respectively distinct even upon the gill plumes.

Shading and light of constant intensity also act upon diverse receptors, since the reactions they induce are spatially separate and quite distinct in character. If the eyes of *C. zebra* are photosensitive, we must conclude that they are not the only photo-receptors concerned in the activating effect of light of constant intensity, as is shown by the behavior of the gill crown. The 'rhinophores' are preeminently sensitive to touch, but are not reactive to light.

Speaking in general terms, we may, then, distinguish at least three classes of differentiated receptors in *Chromodoris*: tactile organs, light-detecting organs, organs sensitive to sudden diminution of light intensity. The absence of morphologically special-

ized nerve terminals in the integument is no objection to this view; physiologically, no other interpretation is possible, and even if free nerve terminals were the only form of peripheral nervous organs they would have to be regarded as coming into association with epithelial cells so specialized as to be capable of differential excitability. The receptor distinctions here found are qualitative in character, cannot be referred to the quantitative differences between the action of the several sources of excitation (light, touch, etc.), and afford no support to the theory of generalized sense organs open to homologous activation by a diversity of means.

#### IV. THERMAL EXCITATION

The relation of thermal conditions to the effectiveness of other sources of activation (mechanical, photic, or chemical), and to the animal as a whole, is of interest in connection with the possible operation of heat and cold as specific activating agents. We have not investigated the possibility that there may occur in *Chromodoris* seasonal variations in thermal sensitivity, the following tests being concerned with individuals collected in shallow water during June and July.

*a.* When placed in sea-water cooled to 15°C., *Chromodoris* remains relatively quiet. Tactile responses are elicited without more than a slight decrease in irritability. During the winter months, however, when kept constantly at a temperature of about 17°, tactile responses are apparently of lower amplitude than during the summer; the animals also move about somewhat less freely.

After two or three minutes subsequent to immersion in water at 10°, the animal remains stationary; responses to touch are very feeble. The gill crown tends to remain partly contracted within its collar. A few general contractive movements of the body are seen when the animal is first suddenly transferred to water of this temperature, but, since they were not evidenced when the water is slowly cooled, down to 10° (ten minutes for the decrease from 26° to 10°), and since they are seen also at 15° when the nudibranch is suddenly transferred to water at that temperature, it is only on the basis of the gill reaction that water at 10° can be held to be stimulating.

At 4° to 5° the 'rhinophores' and the gills remain contracted, usually completely so. The whole body is ordinarily contracted to a considerable extent, after a few preliminary movements of extension and slow longitudinal contraction. After five minutes, tactile responsive-

ness disappears. *Chromodoris* will slowly recover after fifteen minutes' exposure to 4°C. This is probably the lowest temperature which *Chromodoris* can endure for a similar period and yet recover.

At temperatures of 32° to 35°, the genital papilla becomes extended, in some cases immediately; after fifteen to thirty minutes the proboscis is almost invariably protruded. The gills also contract noticeably upon first immersing the nudibranch, but are subsequently extended very fully. The attachment of the foot is rarely accomplished, the animal lying upon its side in an apparently 'exhausted' state. Tactile irritability is very low. In a number of instances the rhinophores could not be caused to retract when the head region was stimulated. *Chromodoris* survives an hour's exposure to sea-water of 32° to 35°.

Immersion in water at 37° to 38° causes the nudibranch to become active for several minutes, but a quiescent state quickly ensues. Tactile reactivity disappears, except on the gills. Some individuals recover from a thirty-minute exposure to this degree of heat.

At 40°, the nudibranch showed writhing movements for one minute, and then became quiet and remained more or less contracted. After two minutes the gills plumes were curled, but later became extended, and were then found reactive to touch. The rest of the body surface seemed insensitive to touch. After twenty minutes the tactile response of the gill became very weak.

At 42°, mild writhing movements were evidenced for two minutes; the gills sometimes became curled, but in any event were quickly expanded. After three to four minutes no tactile responses were obtained from the gills. *Chromodoris* withstands six to ten minutes exposure to this temperature, but does not fully recover.

Immersion in water at 44° leads to rather sharp writhing movements, lasting several minutes, after which the animal is much elongated with the gills expanded. No tactile responses from the gills after three minutes. One individual showed some movement of the foot and body when transferred to sea-water at normal temperature after being for seven minutes at 44°.

Temperatures above 45° usually produce death, accompanied by considerable contraction, within five to ten minutes.

There appears from these tests to be something akin to 'cold' activation at about 10°, at which temperature the gill crown is partially contracted; some general body movements are also exhibited upon immersing the animal in sea-water cooled to this degree. The anaesthetising effect of low temperature is very evident in *Chromodoris*. Noticeable contraction of the gills occurs also at 32°, which represents probably the minimal temperature for a distinct 'heat' stimulus.

b. Change in temperature has an immediate effect upon the rate of the heart beat in *Chromodoris*. The thin-walled heart lies near the dorsal surface of the animal, immediately anterior to the gill crown. Its pulsations can be plainly seen and rather accurately 'timed' in the intact animal. The rate of contraction of the auricle varies greatly in different individuals under closely similar external conditions—from 7.2 to 22 seconds being required for ten beats at 24° to 25°C. in a number of animals examined. The frequency of the beats is readily controlled by the temperature, as may be seen in the following notes of an experiment with one individual:

<i>Temperature C.</i>	<i>Individual A</i>	<i>Seconds for 10 beats</i>
25° (Room temperature).....		22.0
30° after 3 minutes immersion.....		14.4
after 15 minutes immersion.....		14.0
after 20 minutes immersion.....		13.6
after 40 minutes immersion.....		20.5
25° .....		20.5
35° after 5 minutes immersion.....		11.0
after 10 minutes immersion.....		10.5
after 15 minutes immersion.....		10.0

In this experiment the pulsation rate is a little more than doubled by a rise of 10° (25° to 35°), but the relation between rate and temperature is a linear one (fig. 7, *A*), not 'exponential.' Other experiments checked this finding (fig. 7, *B*), so far as the temperature range 20° to 35° is concerned. At temperatures above 35° the heart beat became faint and irregular, following a preliminary acceleration, and hence difficult to count. Below 18° the heart beat could not easily be counted in the intact animal, because of its faintness, but it seemed (see dotted line, fig. 7, *B*) that the straight-line relation between frequency and temperature was departed from at the lower temperatures.

c. In the attempt to localize possible areas of special thermal sensitivity, small volumes of warm water were applied from a pipette to different regions of the animal's surface. The anterior end seemed the most sensitive part, but it was not delicately so. A stream of water running under low pressure from

a heated tank was allowed to flow over various portions of the surface. A delicate thermometer held in a position corresponding to that of the part concerned was used to get some idea of the temperature of the water stream. The current did not stimulate. With sea-water at 40°C., the buccal veil seemed the most sensitive part. The tentacles also gave good responses. The gills likewise reacted locally to water of about this temperature. Not

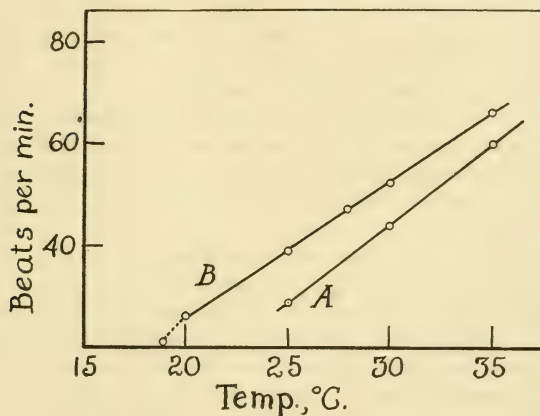


Fig. 7 Relation of the rate of the heart beat to temperature, in two individuals, A and B.

until water was used issuing from the tube at a temperature of nearly 50° were well-defined reactions had from the 'rhinophores,' the tactile irritation occasioned by the stream can hardly have been very important in these experiments, since with the water at a lower temperature issuing at the same rate the rhinophores were not activated.

Efforts made in this way to localize a 'cold' response were quite unsatisfactory. The gills react to water at 9° to 10° by weak contraction.



Sunlight concentrated by a lens of 30-cm. focal length gave a very hot spot of light even when screened by 30 cm. of sea-water. This spot was allowed to fall upon different parts of *Chromodoris*' surface. Even though to one's hand the heat stimulation by this focussed beam was intense, it evoked only local reactions—absolutely restricted to the part stimulated—on all parts except the buccal veil and mouth region. The buccal veil and the tentacles were the most sensitive parts, and they were about equally sensitive, so far as could be judged. The 'rhinophores' gave merely weak homolateral responses, but when the spot of (light and) heat was allowed to fall on the oral area, the animal bent sharply away. Immediately outside the central part of the focussed beam, when not sharply focussed, the heat effect was slight, as tested with a thermometer, but the light cone brilliant; this light did not, however, stimulate the integument of the oral region. Therefore the foregoing responses must have been due to heat.

Experiments were then made in a trough 30 cm. long, containing sea-water and heated at one end. When the water at this end was at 32° to 33°C. and that at the other 25°, a *Chromodoris* was introduced at the cooler end of the trough. The heated end was toward the light coming from a window facing the sun, and the *Chromodoris* tended therefore to move into the region of the warmer water. In seven experiments the nudibranchs tested moved promptly toward the light, but ceased moving, elevated the anterior part of the foot from the substratum, and contracted the buccal area when they encountered water of 31° to 32°.

*d.* These several lines of evidence point to the existence of well-defined, though not very delicate, thermal sensitivity. The limiting temperatures (for 'cold' 10°C. and for 'heat' about 32°C.) are extreme, so far as the normal experience of the nudibranchs is concerned. Nevertheless, under natural conditions, temperatures of 31° are met with in shallow water, and even lower temperatures may have a distinctly directive effect. The temperature responses in *C. zebra* are so vague as to be difficult to study carefully; we cannot say that 'heat' and 'cold' receptors are distinct, but it seems probable that 'heat' receptors are dis-

tinct from tactile sense organs and from photoreceptors. The thermal sensitivity of *Chromodoris* seems distinctly superior to that of *Chiton* (Arey and Crozier, '19).

#### V. CHEMICAL EXCITATION

*a.* The general surface of the body of *Chromodoris* is capable of being sensorially activated by diverse chemical agents (Arey, '17, '18). The dorsal and lateral skin reacts locally to chemical irritation by forming deep pit-like depressions. The 'rhinophores,' oral tentacles, gill plumes, and mantle edge are all open to chemical activation. The 'rhinophores' and oral tentacles are in a general way the most sensitive parts. The reactions elicited from these and other portions of the nudibranch's surface are identical with those to mechanical excitation.

In testing the distribution of chemical excitability, equal amounts of various solutions were allowed to flow from a capillary pipette with its tip at an approximately uniform distance from the surface in each test. Care was used to avoid mechanical stimulation from the stream. About 0.5 cc. of fluid was employed, the nudibranchs being submerged in sea-water.

Rain water or distilled water used in this way affords a weak stimulation everywhere, save on the gill plumes, where it usually fails. The responses are local, never involving movements of the body as a whole. Sea-water diluted with three times its volume of rain-water did not induce any reactions. Sea-water evaporated to half its original volume was effective for excitation; the 'rhinophores' and the head region were noticeably reactive, the rest of the body less so.

Rain-water is, of course, fatal as a medium for the whole animal, which lives about forty-five minutes when completely immersed in it; within fifteen minutes the sensitivity of the 'rhinophores' to mechanical and to chemical excitation is obliterated. The 'rhinophores,' gill plumes, and body musculature contract greatly on first submerging the animal in rain-water, but the 'rhinophores' and gills are subsequently extended. Under the conditions of the following tests, however, osmotic relations are not of primary importance.

b. Substances of the sugar group are quite ineffective for chemical stimulation of the body surface. Maltose and sucrose solutions, 1 M in rain-water, or saturated lactose solutions (0.5 M $\approx$ ) induced no response. Glycerin, 2 M and 3 M, was reacted to by all portions of the surface; more dilute solutions were ineffective; stimulation was in this case of osmotic origin.

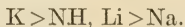
Solutions of several alkaloids: cocaine (0.01 M), nicotine (0.03 M), and weak solutions of curare gave very slight activations; atropine sulphate (0.01 M) was quite stimulating. Solutions of urea (0.1 M) and of urethane (0.005 M) likewise failed to stimulate, although chloretone (0.005 M) gave good responses from all parts. These solutions were made up in sea-water. As a whole, the alkaloids and narcotics were relatively inefficient for activation.

The following substances were found capable of producing sensory activation: neutral salts, various acids, KOH, ethyl alcohol (0.1 M), irritants such as H<sub>2</sub>O<sub>2</sub>, and various essential oils. These substances, in appropriate concentration, led to pronounced reactions involving the whole body.

In the characteristic features of their activation, no less than in the variety of substances to which reaction is given, the generally distributed chemical sense of *Chromodoris* resembles that of other marine animals (Arey and Crozier, '19). Thus in 0.625 M solution in rain-water, the chlorides of the alkalies are effective in the following order:

NaCl	} Mild responses, chiefly from the 'rhinophores' and oral tentacles. The remainder of the general surface but weakly reactive; no clear differences obtainable between the effects of these two salts.	Very weak responses.
LiCl		
NH <sub>4</sub> Cl		
KCl		Extremely strong reactions from all regions.

From the relative vigor of the responses attending stimulation of the mouth region and of the 'rhinophores,' the cation order of efficiency is



The stimulation here is primarily a matter of the cation. KCl, KB, KI, and KNO<sub>3</sub> lead to equally strong reactions from all

parts.  $\text{CaCl}_2$  and  $\text{MgSO}_4$  (0.625 N) did not activate;  $\text{MgCl}_2$  gave fairly good responses over the whole surface of the nudibranch.

The limiting dilutions of different substances effective for activation are likewise characteristic. For any one substance the distribution of limiting effective concentrations for the different parts of the body affords a measure of their respective receptivities. In the following summary it will be seen that the several concentrations, especially for the oral region, are of the orders of magnitude found in the stimulation of other animals:

*Picric Acid*, dissolved in sea-water.

M/150	Good, strong reactions everywhere.
M/200	Gill plumes respond weakly, or merely flatten out against the body wall.
M/500	} Good responses everywhere, except from the gills.
M/1,000	
M/4,000	Oral tentacles still very sensitive 'Rhinophores' less sensitive: they may merely bend before the stream of acid, and not distinctly contract. The dorsal skin is also slightly sensitive
M/8,000	Same as M/4,000, but weaker.
M/10,000	The mantle edge fails to respond; the edge of the foot, especially its anterior part, is still sensitive. Response from the oral tentacles is more constant than from the 'rhinophores.'
This solution is distinctly bitter to human taste.	

*KCl*, in rain-water.

M/700	Gill-plume reaction weak.
M/1,000	No response from the gills.
No responses distinguishable from those to an equal volume of rain-water were obtainable with weaker concentrations.	

In sea-water solution the reactions from the different regions of the body, at M/400 concentration, indicated the following regional order of decreasing irritability (Crozier, '16 a, p. 272):

anterior tentacles, 'rhinophores' > base of the gill > crown > buccal mantle > posterior mantle veil > lateral mantle edge (ventral surface) > edge of foot (at the sides) > dorsal integument.

*KOH*, in rain-water.

M/300	Gill plumes commonly fail to react.
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*KCl*, in rain-water.

M/3	Responses from all parts.
M/16	No responses from gill plumes.
M/50	Doubtful if the responses are distinct from those to rain-water in sea-water.
M/15	Gill plume reactions fail.
M/20	No reactions from any part.



c. From the comparative reactivities of different parts of the body and from the relative limiting concentrations of each substance required for the activation of these parts, the distribution of general chemical sensitivity can be made out over the body surface of *Chromodoris*. These two criteria lead to mutually concordant results, as an inspection of the preceding paragraphs will disclose.

The gill plumes are distinctly the least sensitive of the outgrowths from the body; the oral tentacles probably the most sensitive; the 'rhinophores' almost as sensitive as the oral tentacles, but occupying apparently an intermediate position. On the ground of distribution, it would appear that chemoreception is served by distinct receptors, for the gill plumes are reactive to shading, touch, etc., as already described, in a way which indicates their possession of delicate receptive mechanisms for these sources of activation, yet their chemical reactivity is slight. Evidence of similar import is afforded by comparing the responses of the oral tentacles and of the 'rhinophores.' At elevated temperatures (38°C.), tactile responsiveness is quickly destroyed on all parts, but sensitivity to KCl solution (0.625 M) is preserved. After complete exhaustion to shading, the gills are fully responsive to KCl, M/1,000 picric acid, etc.

The genus *Chromodoris* is characterized by the fact that many, or most, of its members tend to develop a blue or purple pigmentation of the skin. This pigment is a delicate indicator of acidity (Crozier, '14, '16 a), turning pink with acids. This color change is not indicative of an alkaline reaction in the cell interior (Simroth, '14, p. 484), because, although the cell contents are more acid than sea-water, the pigment is still blue under faintly acid conditions (Crozier, '16 a). This natural indicator offers an exceedingly favorable opportunity for studies on the penetrability of cells for acids, leading to the possibility of investigating the nature of the reaction between acid and tissue in the process of stimulation (Crozier '18 a). In the case of neutral salts, we must also suppose that stimulation is due to some chemical influence of the salt upon the surface of the receptive elements, possibly owing to the fact that the applied



salt influences the ionization of protein salts located at these surfaces. The very beautiful experiments of Loeb ('18 a, b) open a way to precise interpretation of this matter. The relative effectiveness of various ions of neutral salts follows an order familiar in many cases of physiological action, frequently regarded as evidence of action upon the colloidal, as distinct from simply chemical, properties of tissue proteins. These effects cannot be interpreted in terms of 'permeability,' since, according to Osterhout's ('16) exact experiments, the influence of neutral salts upon permeability does not follow this plan. Neither for salts nor for acids can stimulation be regarded as due to increased permeability of the cell surface.

d. Those reactions of *Chromodoris* which concern its 'behavior' in the larger sense have to do with feeding and with copulation. The generally accepted idea that the 'rhinophores' are specialized chemoreceptive organs concerned with olfaction has been already disproved (Arey, '17, '18). *C. zebra* does, however, give evidence of being activated by low concentrations of materials secreted by its companions. These reactions are chemopositive, they are of several kinds, and they are important for conjugation. It is also probable that chemoreception enters into food taking, for it is only when creeping upon algae that the radula is brought into operation.

When several sexually ripe nudibranchs are placed in a dish, they very soon protrude the genital papilla, and move toward one another. In fresh sea-water, as, e.g., in an aquarium with running water, conjugation is quickly effected.<sup>6</sup>

These nudibranchs produce constantly, when undisturbed, more concentratedly if irritated (Crozier, '16 b), a curiously penetrating 'spicy' odor. This odor is evident in sea-water with which they have been in contact. If the water is unchanged it assumes a blue color owing to the secretion of pigment. In stagnant water a *Chromodoris* may, but usually does not, protrude the genital

<sup>6</sup> According to Pelseneer ('11), and as we also have observed, many smaller nudibranchs deposit several egg masses following each insemination. In *Chromodoris* this is not true, a single egg mass being the consequence of each insemination (Crozier, '18<sup>d</sup>).

papilla when isolated by itself. In stagnant (i.e., non-circulating) water copulation is interfered with (Crozier, '18 d), although the nudibranchs may be active and healthy. These facts indicate a chemopositive response to low concentrations of some secretion, which is inhibited by higher concentrations. Mating behavior remains the same after the 'rhinophores' have been amputated. Therefore the 'rhinophores' are not of special importance for this response.

Not only is the genital papilla protruded, but the pharynx as well is everted, even before two conjugating individuals come into contact. It is very difficult to experiment with the sensitivity of the pharynx, because it is seldom extruded in a position



Fig. 8 The 'mouthing' behavior of *Chromodoris* preparatory to copulation

favorable for observations, but it is undoubtedly very sensitive both to touch and to chemical activation. To KCl, M/20 in sea-water, the lips of the fully protruded pharynx were found more sensitive than its outer surface, whereas the walls of the organ (section I) were more reactive than the lips to touch. It usually happens that before becoming mutually adjusted for copulation (Crozier, '18 d), the nudibranchs pass the end of the pharynx over each other's surface, moving closer together the while (fig. 8). It seems hardly doubtful that the lips are the chief chemoreceptive regions in this case, because they may be the only parts in contact with the other animal. This form of chemical attraction is curious, because 'sexual' secretions can hardly be involved in a functioning hermaphrodite when reciprocal fertilization

takes place (Crozier, '18 d); still, it is possible, for one of the individuals is commonly more active than the other in the maneuvers preliminary to copulation.

#### VI. SUMMARY

1. Physiological evidence is adduced for the existence in *Chromodoris zebra* of differentiated receptive mechanisms mediating reactions to tactile, chemical, and shading stimulation, to the constant intensity of light, and perhaps to heat.

2. Locally, responses of the general integument and all of the outgrowths of the body, gill plumes, 'rhinophores,' tentacles, pharynx, depend upon locally contained, peripheral, non-synaptic networks. In the gill plumes, and probably in the other projecting parts, these nerve nets are polarized.

3. Reactions involving parts distant from the site of activation depend upon central, ganglionic, transmission. The peculiarities of heterolateral response; of irreciprocal conduction between the several homolateral parts; and of behavior following strychnine injection, show this central nervous system to be essentially synaptic.

4. The nudibranch is positively phototropic, the chief receptive organs probably being the eyes. The branchial collar is also sensitive to light, which causes the gill plumes to be expanded. The gill plumes react by contraction when they are shaded; this response is very variable. When sexually ripe, *Chromodoris* is negatively geotropic. It is negatively rheotropic to strong water currents, the directive organs being the 'rhinophores.' Vibrations transmitted through the water are not responded to. Temperatures of 31° to 32°C. induce negative reactions. Chemotropic reactions to body secretions of other individuals lead to conjugation; 'olfactory' stimulation (which does not essentially involve the 'rhinophores') as well as 'gustatory' stimulation (of the lips) are concerned in this behavior.

5. The locomotion of *Chromodoris* is primarily muscular, not ciliary, the active part being the outer lateral margins of the foot, which suck locally. Progression is strongly polarized in

the anterior direction. The foot is positively stereotropic, and when removed from a surface folds together laterally. These latter peculiarities enable the animal to creep upon narrow blades of eel grass where it feeds. The stereotropism of the anterior end of the foot is responsible for righting behavior; there is no apparent statolythic control of dorsoventral body orientation.

Dyer Island, Bermuda,  
June, 1918.

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Resumen por el autor, Matsuziro Takenouchi.  
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Estudios sobre la supuesta función endocrina de la glándula timo  
(rata albina).

El suero procedente de conejos inmunizados con la substancia del timo de la rata albina presenta una reacción de precipitina positiva con el extracto del timo, pero esta reacción no es estrictamente específica. El suero anti-tímico de conejo no produce ninguna acción hemolítica positiva con los corpúsculos sanguíneos de la rata cuando se usa como complemento el normal del conejillo de indias o el suero de la rata. Los sueros anti-tímicos inyectados en ratas no producen síntomas de "anafilaxia primaria" ni tampoco afectan al crecimiento de estas. El autor no ha observado modificaciones en las vísceras. Ha observado casi los mismos resultados con el suero anti-testicular de conejos inyectados con emulsión de testículo de rata. Ha intentado provocar la producción de hemolisinas en los conejos mediante la inyección de corpúsculos sanguíneos de la rata lavados, pero el suero normal de la rata con su complemento no puede activar la hemolisina contra los corpúsculos sanguíneos de ésta, a causa de la presencia de una substancia inhibidora. El pollo no es animal adecuado para la producción de hemolisina capaz de actuar sobre los corpúsculos sanguíneos de la rata. Nuestro intento de producir suero anti-tímico enérgico en el conejo mediante inyecciones de timo de rata fracasó probablemente por la producción de anticuerpos en el conejo y porque las células del timo de la rata están protegidas. No podemos admitir hasta el presente la existencia en el timo de una función endocrina bien establecida.

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## STUDIES ON THE REPUTED ENDOCRINE FUNCTION OF THE THYMUS GLAND (ALBINO RAT)

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TWO CHARTS

### CONTENTS

1. Introduction.....	311
2. General plan and technique.....	315
3. Immunization of the rabbit with the thymus gland of the albino rat....	317
4. Immunization of the rabbit with testes of the albino rat.....	327
5. Immunization of the rabbit with the red corpuscles of the albino rat....	330
6. Immunization of the chicken with the red corpuscles of the albino rat...	335
7. General discussion.....	336
8. Résumé and conclusions.....	339

### 1. INTRODUCTION

The various theories of the control of sexual development by the thymus, mostly founded upon the correlation in man of the time of thymus involution and sexual differentiation, do not seem as yet to be firmly established. On the one hand, Klose and Vogt ('10), Lucien and Parisot ('10), Paton ('11), and other authors report that following thymus removal in birds and mammals various effects are found, such as interference with skeleton growth, including rachitic changes, together with adiposity or emaciation, injury to the thyroid and degeneration of the testes; while on the other, Pappenheimer ('14), Park ('17), and others state that if the experiments are carefully performed and carefully controlled, the thymus can be removed without producing any harmful effect whatever.

In feeding experiments, Gudernatsch ('14) found that thymus-fed larvae delayed their metamorphosis, although the animals grew on this diet. Romeis ('15) and Abderhalden ('15) were

able to verify these findings in part, but Swingle ('17), Uhlenhuth ('18), and Hoskins ('16) report negative results.

Uhlenhuth in his latest paper ('19), however, states that the metamorphosis of the salamander larva is retarded when thymus gland is fed.

The theory of the relation of the thymus to general metabolism also lacks definite proof (Jackson, 15, '15 a; Stewart, '18), and the theory that rachitic changes in children are caused by disturbances of the thymus cannot be proved by any known facts.

The cells which make up thymic tissue belong to the vascular system (Danchakoff, '16), and Adami ('14) states on page 563 of his text-book that "To all intents and purposes it (the thymus) is a lymphoid organ," and the formation of an internal secretion is no more likely to be the function of the thymic cells than of the cells of similar appearance in other lymphoid tissues. Summing up all the physiological, anatomical, and experimental facts, E. R. Hoskins ('18) says that whatever be the real function of the thymus, certain it is that the production of an internal secretion by it has not been proved.

Among other experiments with the thymus which cannot be placed under the headings, extirpation or administration of thymic tissue or extracts, there are two; one, which aims to destroy the thymic tissue in vivo by x-ray irradiation, while the other seeks to do the same by some serological method. To the first category belong the experiments of Regaud and Crémieu ('12). They made the thymus atrophic, especially the cortical portion of it, by x-ray irradiation, but they did not observe any abnormality in the health and growth of the animals so treated. Hewer ('16), however, reports that injury to the thymus by x-ray irradiation results in injury to the function of the testes. "Since complete removal of the thymus has no such effect, and since unhealthy animals never breed well, Hewer must prove that her treatment did more than injure the health of her animals" (Hoskins, '18).

Under the second category, namely, the action of thymotoxic or thymolytic serum, there are many reports on record. Gilberti



('11) used rabbits for immunization to obtain a thymotoxic serum, with positive results when dog thymus extract was employed as the antigen. "Weymersch ('08) sah nach Injektion von thymotoxischen Serum eine weitgehende Atrophie und Sklerosierung der Thymusdrüse, eine abnorme Verteilung der Leucocyten und exzessive Wachstum der Thiere" (cited from Shimizu's paper, p. 262). Ritchie ('08) obtained a serum from ducks by injection of the thymus of guinea-pigs, but observed no specific action of that serum, when injected, on the thymus of guinea-pigs. He states, however, that in the presence of this immune serum from ducks, guinea-pig complement becomes fixed to the guinea-pig thymus, lymph glands, bone marrow, and spleen. Ritchie used in his experiment a hemolytic system consisting of ox blood and anti-ox rabbit serum. He concludes from this experiment that the serum obtained from ducks which had been treated with the thymus glands of guinea-pigs contained a 'leucophilic immune body,' and not a specific thymolytic one, and that the structural changes in the thymus of the guinea-pigs following the injection of the serum were due to its 'leucolytic action,' and are not specific.

Moorhead ('05), however, found that the serum of rabbits which had been injected with guinea-pig's thymus glands had no recognizable leucolytic action, did not "agglutinate emulsified thymus gland in vitro," and had no constant action upon the animals into which it was injected.

Recently Shimizu ('13) reports his success in obtaining a very strong thymolytic serum, which he says resulted, after injection in young dogs, in a marked retardation of bone growth, together with a strong atrophy of the medullary portion of the thymus, and a proliferation of connective tissue. These responses occurred in two animals among fifteen so treated, while there were in all the other individuals more or less pronounced toxic symptoms corresponding to the so-called primary anaphylaxis. In the summary of his paper he says:

Bekanntlich ist die Veränderung der Thymus bei Inanition hauptsächlich die Involution der Rindensubstanz, und das Mark bleibt dabei wohlbehalten. Thymusatrophie bei der Röntgendurchstrahlung

betrifft auch die Rinde. Die bei den Ernährungsstörungen und akuten Infektionskrankheiten der Säuglinge vorkommende Veränderung der Thymus ist auch eine Rindenatrophie. Wie Hammer betont hat, sieht man eigentlich nur die Rindenatrophie sowohl bei der Alters—, als auch bei der durch Hunger, toxische Einflüsse etc. hervorgerufenen akzidentellen Involution. . . . Mein Thymolysin ruft hauptsächlich deutliche Atrophie des Marks hervor, und die behandelte Tiere zeigen dieselbe körperlichen und geistigen Entwicklungen, die von den früheren Autoren bei Thymektomie beobachtet worden sind.

From the result of his experiments he concludes that the medullary portion of the thymus has biologically a distinctly different function from the cortical portion, and that the endocrine function of this gland with its influence upon the growth of animals, must be ascribed to the medullary portion only. He does not give definite proof for any endocrine function whatever of the thymus, at least in his first paper,<sup>1</sup> and he made his conclusion with the complete acceptance of the theory of the thymus-skeleton relationship, which had already been firmly established in the minds of many.

As is easily imagined, it would be one of the best methods for the study of the function of the thymus gland to destroy it alone *in vivo* by some other than surgical means, for instance, by some serological procedure, and observe the symptoms or pathological alterations which might follow.

Shimizu does not give any definite proof for the presence of his thymolysin *in vitro*. Also he does not give any histological description of the lymphoid tissues of dogs injected with his thymolysin. This is important, because the cells which make up the thymic tissue belong to the vascular system, according to Danchakoff ('16), and therefore we might expect the lymphoid tissues to respond in some way or other to the thymolysin.

Originally I hoped to repeat in full Shimizu's experiments, using albino rats instead of dogs, and thus to get further information regarding the function of the thymus in the albino rat.

<sup>1</sup> It is said that Shimizu has published his second paper regarding the same problem, and some other Japanese investigators have done work in Japan along this line and published their results. We are, unfortunately, not able to obtain these original reports in time, therefore this paper is written without the knowledge of their publication.

The first thing needed is to verify the fact that, according to Shimizu, the thymolysis in vivo can effect more than the total removal of the thymus gland which, if carefully done, can be accomplished without any harmful effect whatever Pappenheimer, '14, and Park, '17).

## 2. GENERAL PLAN AND TECHNIQUE

For the study of any cytolsin the first condition is to obtain a good antibody (amboceptor, cytolsin) of high potency, and the second condition is that the cytolsin thus obtained should show its action clearly in the body of the same kind of animal as that from which the antigen for the immunization has been obtained, utilizing the complement of that body, or at least it should be able to combine with the complement of some animal in vitro, to show its action clearly.

To choose a proper combination of species (one animal for the source of antigen, the other into which the antigen is to be injected to obtain a cytolytic serum of high potency), is sometimes difficult without preliminary experiments, because the ease with which different species produce strong antibodies after injection of a given antigen is very variable.

For the second condition mentioned it should be kept in mind, as Bordet ('06) says, "*que la valeur h mo- ou bacteriolitique des alexines varie d'une esp ce   l'autre, rein de plus admissible, les d'esp ces differente n' tant pas enti rement identique.*"

In general for hemolysis, fresh guinea-pig serum is very potent in activating many sensitized blood-cell complexes, but weak in activating sensitized guinea-pig corpuscles. Often we find that the complement from an animal is entirely impotent or capable of producing only a weak hemolysis of the sensitized cells of its own species, though this is not a general rule (Zinsser).

The usual technique was employed. For the immunization of the rabbit with thymus we used the thymus from albino rats taken from the stock colony of The Wistar Institute. Rats were chosen between eighty to ninety days, which, according to Hatai ('14), is the period when the thymus gland is largest in the albino rat.

Under ether narcosis the blood of the rats was taken from the arteria carotis and the thymus gland was removed under aseptic precautions, avoiding the mixing of blood as completely as possible. After being weighed, the glands were washed in sterile saline solution to free them from visible traces of blood and then were ground with sterile salt solution into an emulsion, which was strained through sterile cotton gauze and injected into the rabbit intraperitoneally.

The injection was repeated three or four times with increasing doses of the thymus emulsion, at about one week's interval, and seven to ten days after the last injection the blood was taken under ether narcosis from the arteria carotis. Autopsy findings were noted. The separated serum was carbolyzed (0.5 per cent) and kept in the ice-box.

The normal serum which was used in the control experiments was taken from the ear vein of a normal rabbit without narcosis, carbolyzed as above, and also kept in the ice-box.

For the detection of antibodies *in vitro*, we used the precipitin reaction with the extracts of several organs from normal rats. The technique used in the preparation of the extracts will be given later.

For the determination of antibodies *in vivo*, we injected the antithymus serum repeatedly into albino rats, using generally 0.3 cc. to 0.5 cc., injecting subcutaneously on the back near the tail, and observing any marked symptoms which followed. Generally one half of one litter of rats was used as the test animals, and the other half as controls. All the rats were fed with ordinary laboratory diet. The growth curve was obtained by weighing each rat separately.

The examination of the test and control rats followed at different intervals after the last injection of the serum. Under ether narcosis, blood was collected, each organ removed separately and weighed carefully and, directly after the weighing, all the organs were fixed in Bouin's solution for histological study.

For the comparative study on the specificity of cytolytic serum, I also immunized rabbits with testes from albino rats, using almost the same technique as in the immunization with

thymus tissue. The serum thus obtained was tested in vitro as well as in vivo.

For the further serological studies, a preparation of hemolytic rabbit serum against the red corpuscles of the albino rat was attempted, and, furthermore, chickens were immunized against rat blood, and the action of the normal rat complement carefully examined in these cases, using the ordinary serological technique, which will be given later.

### 3. IMMUNIZATION OF THE RABBIT WITH THE THYMUS GLAND OF THE ALBINO RAT (RABBIT, GROUP A)

#### *a. Process of immunization*

A large, healthy female rabbit was immunized with the thymus substance of albino rats by intraperitoneal injections.

##### *Rabbit, Group A, No. 1*

<i>Date of injection</i>	<i>Amount of thymus emulsion injected</i>
June 25, 1918.....	2.75 grams of thymus.
July 1, 1918.....	3.10 grams of thymus.
July 8, 1918.....	4.12 grams of thymus.
July 18, 1918.....	4.5 grams of thymus.
July 27, 1918.....	The blood was taken.

The autopsy findings were as follows: Spleen somewhat smaller than usual, without marked macroscopical alteration, liver normal; kidneys both normal. On the right side, in the middle part of the abdomen, directly inside of the peritoneum, there was a small fibrinous body about 12 mm. in length, 6 mm. in breadth, which is nothing else than an abscess enclosed in a strong fibrinous membrane hanging between the convolutions of the intestine. This abscess was most probably caused by the injection of the thymus emulsion, which though strained, had in it some small particles of connective tissue which were dissolved with difficulty.

The separated serum was tested for the presence of the antibodies by the precipitin and hemolytic reactions and then injected into rats to determine the action in vivo.



*b. Test of the antithymus serum in vitro*

1. *Precipitin reaction.* For the precipitin reaction, we followed the procedure given by Ricketts and Rothstein ('03) for the action of neurotoxic serum. They used for their precipitin reaction an emulsion of nervous tissue as a precipitinogen (antigen).

Different precipitinogens (antigens) from different kinds of tissue were prepared in the following manner:

Weighed tissue, first washed with saline solution, was ground thoroughly, emulsified with sterile saline solution and shaken six hours at room temperature, with preliminary carbolization for the purpose of avoiding bacterial contamination and alterations in composition. Then it was strained through sterile cotton gauze. The strained emulsion was centrifuged for a long time at high speed to free the supernatant fluid from macroscopic particles. The supernatant fluid was diluted again with sterile saline solution and carbolized. The following tissues were prepared in this way.

TABLE 1

	TISSUES				
	Kidney	Spleen	Testes	Brain	Thy-mus
Weight (grams).....	2.75	0.842	0.742	1.500	0.899
Salt solution added in grinding (cc.).....	15.0	5.0	5.0	10.0	5.0
Salt solution added to the supernatant fluid (cc.).....	30.0	10.0	10.0	20.0	15.0

To six test-tubes each containing 1 cc. of the diluted extract there were added from one to five drops of serum, the sixth test-tube being held as control. The quantity of the liquid in each test-tube was made the same with saline solution. The tubes were then put into the ice-box for sixteen to eighteen hours, avoiding any evaporation. The first reading was made after four to six hours and the second sixteen to eighteen hours later (table 2).

Control tests with normal rabbit serum give a little precipitation in some tubes, but this is slight and inconspicuous in others.

TABLE 2

*Showing the precipitin reaction of the antithymus serum with the extracts of different rat organs*

	TEST-TUBE					
	1	2	3	4	5	6
Extract (cc.).....	1.0	1.0	1.0	1.0	1.0	1.0
Antithymus serum in drops.....	1	2	3	4	5	
First reading (4 to 6 hours)						
Kidney.....	—	—	—	—	—	—
Spleen.....	—	—	—	—	—	—
Testes.....	—	—	—	±	++	—
Brain.....	—	—	—	—	—	—
Thymus.....	+	+	++	++	++	—
Second reading (16 to 18 hours)						
Kidney.....	+	++	++	++	++	—
Spleen.....	+	+++	+++	+++	+++	—
Testes.....	+	++	+++	+++	+++	—
Brain.....	—	—	—	—	—	—
Thymus.....	++	+++	+++	+++	+++	—

+++ , marked sedimentation.

++ , less marked, but easily recognizable precipitation.

+ , slightly positive precipitation.

— , negative precipitin reaction.

Another technique for the precipitin reaction which is generally used in serological work (namely, mixing the antiserum with about an equal amount of some dilution of antigen injected for the production of the antiserum, with resulting turbidity and rapid flocculation) was tried with the material used in the above tests, but the reaction was inconspicuous in all cases.

Using as precipitinogen, serum from a normal rat, diluted ten times, instead of organ extract, all the test-tubes, to which from one to five drops of the antithymus serum had been added, failed to show either precipitation or sedimentation.

From the result mentioned in table 2 we can tentatively conclude that the antibodies which are present in the antithymus serum are not so specific as we are taught to believe by some

investigators. A similar lack of specificity of the antibody in the antiserum produced by injection of rat testis tissue to the rabbit will be mentioned later.

2. *Test of the hemolytic power of this antithymus serum.* The antithymus serum from the rabbit does not possess any appreciable amount of hemolysin against the red corpuscles of the rat in vitro. For the test in vivo, 1 cc. of that serum was injected subcutaneously into three rats thirty days of age, taking three more rats of the same litter as controls. All three rats grew perfectly well and reached normal size. Just the same effect was observed with the antithymus serum from the second and third rabbits (rabbits B and C).

*c. Test of the antithymus serum in vivo*

Three litters of healthy young rats of the same age were taken for this test (series no. I, II, and III). To one half of each litter antithymus serum (from 0.3 cc. to 0.5 cc.) was injected subcutaneously, while to the other half of each litter corresponding amounts of normal rabbit serum were injected. All the rats were put under the same conditions and the growth was controlled by weighing each rat almost every day. Six to twenty-five days after the last injection, some of the rats, both test and control, were examined for the weights of each organ.

Histologically, studies of the thymus gland from all the rats were carefully made to see whether there was any histological change caused by the injection of the antithymus serum.

As an example of the growth of the test and control animals, I will give one chart which shows the average curve of the rats in series II (chart 1).

In no case of the injection of either the antithymus serum or the normal rabbit serum did we observe any pathological symptoms like the so-called primary anaphylaxis due to the serum injection, or any sudden decrease of the body weight of rats, such as those reported by Shimizu ('13) in his dogs, after the injection of antithymus rabbit serum.

The slight fluctuations of body weight in our charts depend very much upon the amount of stomach contents of the rats. Though the weighing was done mostly at a definite time in the morning before the regular feeding of the rats, circumstances sometimes obliged us to weigh our rats at other times. In any

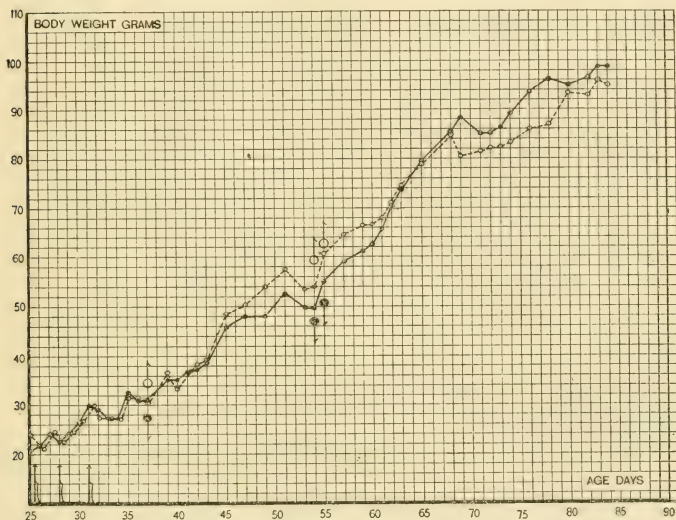


Chart 1 The average growth curve in body weight on age, series 11. The number of test rats, 4; the number of control rats, 5. The inverted black-dot arrows ( $\downarrow$ ) denote the injection of the antithymus serum into the test rats. The ring arrows ( $\circ$ ) indicate when each control rat was taken for examination.

case, all the individuals in a litter were weighed at the same time. A special analysis of the weight of the thymus gland of all rats is worth recording here (tables 3, A, and 3, B). The calculated values are based on table 72 in *The Rat* (Donaldson, '15).

The data on the weight of the other organs do not show any definite difference between the test and control animals, and therefore are omitted entirely.

TABLE 3, A

*Showing the details regarding the studies on the weight of the thymus of the test rats*

AGE AT THE TIME OF EXAMINATION	EXAMINED AFTER THE THIRD INJECTION	LITTER NUMBER AND SEX	INITIAL WEIGHT	FINAL WEIGHT	OBSERVED WEIGHT OF THYMUS	OBSERVED WEIGHT OF SEX GLAND	$\frac{\text{OBS. THY. W.}^2}{\text{CAL. THY. W.}} \times 100\%$
<i>days</i>	<i>days</i>		<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>per cent</i>
35	4	I, 3, M.	21	36	0.073	0.123	73
36	5	II, 2, M.	24	38	0.091	0.159	87
40	9	III, 3, M.	22	33	0.062	0.236	59
41	10	I, 1, M.	22	43	0.125	0.382	102
54	23	II, 4, F.	19	45	0.067	0.007	49
55	24	II, 3, M.	21	61	0.101	0.597	56
58	27	III, 2, M.	21	57	0.100	0.423	59
64	33	III, 5, F.	23	71	0.173	0.022 <sup>1</sup>	81
64	33	III, 6, F.	22	64	0.157	0.011 <sup>1</sup>	83
83	52	I, 4, M.	21	120	0.200	1.689	70
84	53	II, 1, M.	22	95	0.236	0.764	94
84	53	II, 5, F.	21	102	0.129	0.074	49
84	53	II, 6, F.	18	99	0.217	0.077	85
85	54	III, 1, F.	23	101	0.250	0.071	96
85	54	III, 4, F.	22	94	0.244	0.067	98
85	54	I, 2, M.	20	114	0.266	0.716	98
Average.....							77

<sup>1</sup> The weight of the ovaries of these two rats, of same age, differ as 2:1. This kind of difference may be caused often by ovulation and by the formation of corpus luteum.

<sup>2</sup> The calculated values are based on table 72, *The Rat* (Donaldson, '15).

*d. Repetition of the same plan of experiment with the second and the third antithymus serum (rabbit, group A)*

The same plan of experiment just described was repeated with the second and third antithymus serum, and the respective data will be given here briefly.

The second antithymus serum was obtained by the immunization of a healthy male rabbit (rabbit, group A, no. 2) with the thymus of albino rats, beginning with an injection on August 27, 1918. The injection was repeated three times until the blood was taken September 21, 1918. The autopsy of this rabbit showed nothing pathological. The separated serum, carbolyzed as usual, was used for the experiments.



TABLE 3, B

*Showing the details regarding the studies on the weight of the thymus of the control rats*

AGE AT THE TIME OF EXAMINATION	EXAMINED AFTER THE THIRD INJECTION	LITTER, NUMBER AND SEX	INITIAL WEIGHT	FINAL WEIGHT	OBSERVED WEIGHT OF THYMUS	OBSERVED WEIGHT OF SEX GLAND	OBS. THY. W. CAL. THY. W. $\times 100\%$
<i>days</i>	<i>days</i>		<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>per cent</i>
35	4	I, 2, M.	20	32	0.054	0.152	67
36	5	II, 4, M	24	43	0.071	0.294	59
40	9	III, 5, M.	22	37	0.048	0.276	48
41	10	I, 1, M.	23	46	0.147	0.283	109
54	23	II, 2, F.	18	48	0.195	0.010	139
55	24	II, 3, M.	18	59	0.090	0.600	51
58	27	III, 4, M.	22	60	0.121	0.181	67
64	33	III, 1, F.	23	72	0.162	0.020	76
64	33	III, 3, M.	24	73	0.193	0.516	90
83	52	I, 4, M.	20	121	0.282	1.100	99
84	53	II, 1, F.	23	96	0.231	0.050	91
84	53	II, 5, M.	19	95	0.187	1.193	73
85	54	III, 2, F.	22	111	0.305	0.072	111
85	54	I, 3, F.	21	109	0.190	0.068	70
85	54	I, 5, F.	20	99	0.211	0.043	83
Average.....							82

Precipitin reaction tests with the extracts of several rat organs gave just the same results as were obtained with the first antithymus serum.

For the experiment with the second antithymus serum, three litters of twenty-eight-day rats were used (series no. X, XI, and XII). The injection of the antithymus serum was made on twenty-eighth (0.3 cc.) and on the thirty-sixth, forty-second, and forty-seventh days of age (each 0.5 cc.). Into the control animals, representing almost one half of each litter, nothing was injected. The average growth curve is given (chart 2).

Owing in part to the parasitization of the rats by mites (*Lelaps echidninus*) and to the effects of dipping to kill these parasites, the growth curve of the rats of all three series was very low as compared with the standards (Donaldson, '15), especially in the latter part of October. We observed a sudden drop of body weight of the rats in series no. XI between October 31 and No-

vember 1, 1918, when the examination was made. This was due to the 'dipping' of the rats in soap-kerosine emulsion on the afternoon of October 31st, just after the body weight had been taken. This matter must be taken into consideration in the interpretation of the histological findings in various organs of all rats in these series.

The third antithymus serum was obtained from one rabbit (rabbit, group A, no. 3) immunized by four injections of rat thymus emulsion, beginning October 7, 1918, and the blood was taken on November 8, 1918. The serological tests on precipitin

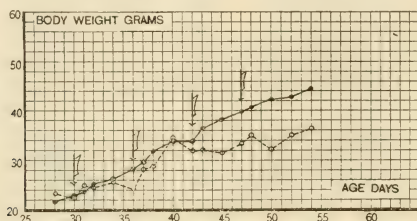


Chart 2 The average growth curve, series no. X, XI, and XII. The graph with dots marks the test series and the arrows show the age at which injection was made. The graph with circles marks the controls.

and hemolysin yielded the same results as in the cases of the first and second serum.

Three litters of albino rats at the age of fifty days were taken for the experiments. The antithymus serum was injected into one half the members of each litter five times, with several days' interval, while into the other half nothing was injected (controls).

The relation of the weight of the thymus of all these rats to the body weight and to the age at the time of examination is given in table 4. The computed relative values for the thymus given in the last column of the table are based on the standard values in table 72, of *The Rat* (Donaldson, '15).

TABLE 4

Showing the details regarding the studies on the weight of the thymus of the test and control rats of series No. XIII, XV, and XVI. C = controls

AGE AT THE EXAM- INATION	EXAMINA- TION AF- TER THE LAST INJECTION	LITTER, NUMBER AND SEX	INITIAL WEIGHT	FINAL WEIGHT	OBSERVED WEIGHT OF THYMUS	$\frac{\text{OBS. THY. W.}}{\text{CAL. THY. W.}} \times 100\%$	
days	days		grams	grams	grams	per cent	per cent
77	10	XIII, 1, F.	34	70	0.161	56	
77	10	XIII, 2, F.	30	72	0.223	78	
77	10	XIII, C, 1, F.	28	75	0.168		59
77	10	XIII, C, 2, F.	30	68	0.128		45
77	10	XIII, C, 3, F.	30	69	0.161		56
87	13	XV, 1, M.	50	105	0.133	46	
87	13	XV, 2, F.	59	95	0.131	46	
87	13	XV, 3, F.	52	75	0.108	36	
87	13	XV, C, 1, F.	48	65	0.101		39
87	13	XV, C, 2, F.	55	100	0.141		49
95	25	XVI, 1, F.	43	120	0.220	82	
95	25	XVI, 2, F.	35	66	0.145	53	
95	25	XVI, 3, M.	43	105	0.132	49	
95	25	XVI, C, 1, F.	32	69	0.162		60
95	25	XVI, C, 2, M.	32	89	0.149		55
95	25	XVI, C, 3, M.	40	113	0.131		48
Average, test rats .....						56	
Average, controls .....							52

*e. General review of the experiments with the antithymus serum*

Looking through the charts which indicate the growth of all the rats used in these experiments, we are convinced that the antithymus sera do not produce any distinct effects on growth. There is no definite difference between those rats injected and those which were used as controls, some of the latter having been injected with normal rabbit serum, while others received no injection at all.

Similarly, the studies on the weights of individual organs do not show any special action of the antithymus serum. The

TABLE 5

*Percentage of the observed weight of the thymus compared with the weight calculated on the body weight and on the age of the test and control animals.*

*Average values*

	CALCULATED ON THE BODY WEIGHT (DONALDSON, '15)		CALCULATED ON THE AGE (DONALDSON, '15)	
	Test	Control	Test	Control
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Series no. I, II, and III.....	77.3	82.0	67.5	73.1
Series no. XIII, XV, and XVI.....	66.0	64.7	55.8	51.5

thymus gland of the test rats, which might have been modified in some way or other by the injected antithymus serum, do not differ in weight from the glands of the controls. This is shown in table 5.

Similar studies made on the weight of other organs of the rats do not indicate any specific action of the antithymus serum.

Though I did not attempt to measure the long bones, inspection indicates that there is no distinct difference in the growth of the bones between the rats injected with the antithymus serum and the controls—a statement which is supported by the relation of the body weight to the body length.

Thus far we conclude that in growth in body weight, in the weight of the thymus, and in the body and tail length, the test and control animals do not show distinct differences from each other. We have not observed any particular anaphylactic symptoms in our animals, such as have been described by Shimizu in his experiments with dogs, after the injection of the antithymus serum; and in general are unable to see any specific action of the anti-thymus serum *in vivo*, so far as the results of macroscopical examination are concerned.

*f. Histological examination of the thymus glands of test and control rats*

After weighing, all the organs were fixed in Bouin's solution. The upper portion of both lobes of the thymus of each rat was used for serial paraffin sections which were stained with haematoxylin and eosin.

The thymus glands of the test, as well as the control animals, were carefully studied. We did not find, however, any regular histological changes which might be attributed to the antithymus serum injection. The dimension of each lobule, the proportion of both cortical and medullary portions, and other findings are similar in both the test and the control rats.

In both test and control rats of series X, XI, and XII, we find a rather high degree of atrophy of the thymus, as is shown in the weight, and the sections also show some peculiarities: atrophy of both medullary and cortical portions, and the presence of some round bodies similar in construction to the so-called Hassel's bodies of some animals.

These latter are located in the middle part of the medullary portion. The diameter of such bodies varies from  $28\mu$  to  $34\mu$ . In construction such a body looks very much like the round bodies in the epithelioma tissue described by Roffo ('18) in his paper on the transmission of epithelioma in the white rat (also Moorhead, '05). Although this may be a pathological change in the thymus tissue, yet it is not caused by the injected antithymus serum, because the same structures are found in the thymus tissue of the control rats also. We are inclined to attribute this histological change in the thymus to malnutrition or to some other condition from which the three litters of series X, XI, and XII suffered, as already described during the experimental period.

Summing up the histological findings, we may conclude that we do not find any alteration to be attributed to the antithymus serum injection.

#### 4. IMMUNIZATION OF THE RABBIT WITH TESTES OF THE ALBINO RAT (RABBIT, GROUP B)

For the purpose of comparative study on the specificity of the cytolytic serum against rat thymus, I attempted to get another kind of cytolytic serum, and for this purpose immunized rabbits against the testis tissue of albino rats.

One large male rabbit received four injections of testis emulsion in physiological saline solution as follows:



<i>Date of injection</i>	<i>Rabbit, group B, no. 1</i>	<i>Amount of testis emulsion injected</i>
July 18, 1918.....		4 testes from two albino rats
July 23, 1919.....		6 testes from three albino rats
August 1, 1918.....		6 testes from three albino rats
August 23, 1918.....		8 testes from four albino rats
September 1, 1918.....		The blood was taken

The emulsion of testes was prepared by grinding the testes in a mortar with physiological saline solution. This was strained through cotton gauze to free it from the particles of connective tissue.

The emulsion was prepared each time just before the injection, so that we did not need to make use of any disinfectant to mix with the emulsion for conservation.

The autopsy of the rabbit showed no pathological changes. The serum was separated carefully and preserved with carbolic acid (0.5 per cent) and used for the tests in vitro as well as in vivo.

#### *a. Test of the antitestis serum in vitro*

The precipitin reaction of the antitestis serum with extracts of several rat organs was tried with the results given in table 6. The technique used in the preparation of the extracts and the method for the precipitin test were those used in the test of the antithymus serum, and therefore the description may be omitted here.

TABLE 6

*Precipitin reaction of the antitestis serum with the extracts of different organs of the albino rat*

	1	2	3	4	5	6
Extract.....	1.0	1.0	1.0	1.0	1.0	1.0
Antitestis serum, in drops.....	1	2	3	4	5	—
Result after 6 to 7 hours in ice-box						
(1) Kidney.....	—	—	—	+	++	—
(2) Testes.....	++	++	+++	+++	+++	—
(3) Thymus.....	±	+	++	+++	+++	—
(4) Spleen.....	—	—	±	++	+++	—
(5) Brain.....	—	—	—	—	—	—

Control tests with normal rabbit serum and different kinds of extracts were tried carefully. Precipitation took place in some test-tubes, but in a decidedly slighter degree than with the immune serum.

*b. Test of the antitestis serum in vivo*

Two litters of the same age, five and six individuals in each litter, were used to test the antitestis serum in vivo (experiment series nos. VIII and IX). About one half of the rats were injected subcutaneously with the antitestis serum, 0.3 cc. to 0.5 cc. at each injection, with five to six days' interval, while the other half, the controls, were injected with the normal rabbit serum. They were put under the same experimental conditions, each litter being in one cage and weighed almost every day until killed. The examination was made in one series at thirty-four days, in the other at forty-six days after the last or fifth injection. Several organs were removed, examined, weighed, and fixed in Bouin's solution for histological examination. In the male special attention was paid to the sperm cells, which were carefully studied microscopically after a quick operation.

Neither the graphs nor the tests just described reveal any particular difference between the test and the control rats, nor is there any marked difference in the weight of the thymus. Table 7 will give the general features of the results.

TABLE 7

*Average percentage<sup>1</sup> of the observed weight of the thymus compared with the weight calculated on the body weight or on the age (Donaldson, '15) of the test and control animals of the series nos. VII and IX*

	CALCULATED ON THE BODY WEIGHT	CALCULATED ON THE AGE
	<i>per cent</i>	<i>per cent</i>
Test rats, 6 individuals .....	63	43.5
Control rats, 5 individuals.....	63.5	38.6

<sup>1</sup> Percentage =  $\frac{\text{Observed weight}}{\text{Calculated weight}} \times 100$  per cent.

Histological examination does not reveal any difference in the structure of the thymus or testes of the test and control rats. The spermatogenesis in the injected rats does not indicate any specific action of the injected antitestis serum.

#### 5. IMMUNIZATION OF THE RABBIT WITH THE RED CORPUSCLES OF THE ALBINO RAT (RABBIT, GROUP C)

Since the experiments on the action in vitro, as well as in vivo, of the antithymus and antitestis sera showed almost entirely negative results, and since it became evident that the production of antibodies in the rabbit against the tissues of the albino rat is very faint, I tried to immunize a rabbit against the blood-cells of the albino rat, because the hemolytic test is rather easier than any other in vitro and the reaction of normal rat complement could be determined more definitely in this way.

##### *a. Process of immunization*

Although, as already noted in the immunization of rabbits with the different organs of the rat, the hemolysin production in the rabbit by the injection of rat organs, or even by the injection of rat erythrocytes, is very faint, nevertheless I tried once more to make sure of this matter.

A large adult female rabbit (rabbit, group C, no. 1) was given an injection of rat corpuscles (0.5 cc. of 10 per cent suspension in physiological salt solution) on October 31, 1918, intraperitoneally. The rabbit did not show any abnormal sign immediately caused by the injection. On November 8, 1918, a double dose, 1 cc. of 10 per cent suspension of rat corpuscles was injected intraperitoneally. After one and a half hours the rabbit became very weak and died shortly in convulsions. The autopsy did not show the immediate cause of death. The blood was removed, however, directly from the heart and vena cava inferior at the upper region of the liver, and the clear serum separated.

This serum was tested for its hemolytic power against rat corpuscles with an entirely negative result. Hemolytic action

of the same, but inactivated serum, using rat serum as complement against rat corpuscles, was tested with an equally negative result.

We can see, therefore, that the hemolysin produced in the rabbit by the injection of a suspension of rat corpuscles is very small in amount, if any, in the first week after the first injection. Unfortunately, the rabbit was killed by the second injection of 1 cc. of 10 per cent suspension, which seems to have been too large a dose for a single injection.

A large, well-developed male rabbit (rabbit, group C, no. 2) was injected with rat corpuscles in smaller doses many times repeated within several days and the blood tested for its hemolytic activity against rat corpuscles.

*Immunization of a rabbit (rabbit, group C, no. 2) against the rat blood*

<i>Date of injection</i>	<i>Amount of erythrocytes injected intraperitoneally</i>
November 12, 1918.....	5 cc. of 5 per cent suspension
November 19, 1918.....	5 cc. of 10 per cent suspension
November 25, 1918 <sup>1</sup> .....	5 cc. of 10 per cent suspension
December 1, 1918.....	Sample blood taken

<sup>1</sup> Before this injection some blood was taken for a sample hemolytic test.

*b. Hemolytic test of the serum in vitro*

1. *Hemolytic test of the sample blood taken just before the third injection.* Active immune serum was tried for its hemolytic power against rat corpuscles. The result is given in table 8.

2. *Hemolytic test with normal rat serum or normal guinea-pig serum as complement.* Hemolytic test of the inactivated antirat blood rabbit serum, using normal rat serum as complement (1)

TABLE 8  
*Hemolytic test of the active immune rabbit serum*

	1	2	3	4	5
Active rabbit serum.....	0.5	0.4	0.3	0.2	—
0.85 per cent NaCl solution.....	—	0.1	0.2	0.3	0.5
2 per cent blood suspension.....	0.5	0.5	0.5	0.5	0.5
Hemolytic result after one hour of incubation and two hours' standing in the ice-box....	+++	+++	++	±	—

and normal guinea-pig serum (2) was conducted in the following manner (table 9).

As is indicated in table 9, we can activate the hemolysin in the antiserum by using the normal guinea-pig serum as complement, while normal rat serum cannot do this, even when a double amount is used. We conclude, therefore, that the normal complement of rat serum cannot be used for activation (complementation) of the hemolysin against rat corpuscles obtained from the rabbit, and this is certainly not because of its quantity, but because of the quality of the normal rat complement. We can make this statement because, according to Kolmer, Yui and Tyau ('13), the activity of the gray rat complement in activating the antihuman amboceptor is about one-third that of the guinea-pig complement, and the activity of albino rat complement is a little stronger, being on the average about half that of guinea-pig complement.

The negative hemolysis in the case of the normal rat serum used for complement may therefore be due to the presence of either, 1) some anti-amboceptor in the rat serum, or 2) of some anticomplementary substance, or to both of these factors.

TABLE 9

*Showing the hemolytic reaction of the immune rabbit serum with normal rat serum or normal guinea-pig serum as the complement*

	1	2	3	4	5	6	7	8	9
Inactive rabbit serum { -1: 5...	0.5	0.4	0.3						
+1: 10...				0.5	0.4	0.3	0.2	-	-
0.84 per cent NaCl solution.....	-	0.1	0.2	0.3	0.1	0.2	0.3	0.5	1.0
(1) Normal rat serum 1: 10	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	-
or									
(2) Normal guinea-pig serum 1: 10									
Results <sup>1</sup> when the normal rat serum is used as complement									
(1).....	-	-	-	-	-	-	-	-	-
Result when the normal guinea-pig serum is used as complement (2).....	+++	+++	+++	+++	++	++	+	±	-

<sup>1</sup> The reading was made after one hour of incubation and six hours in the ice-box.



3. *Determination of the hemolysis inhibiting power of the normal rat serum.* To determine whether the substance or substances in normal rat serum act as a kind of anti-amboceptor or anti-complement, the following tests were made (table 10):

TABLE 10

*Showing the details in the determination of the inhibiting power of normal rat serum on the hemolysis of rat corpuscles by the immune antiserum*

	1	2	3	4	5	6	7	8	9	10
Normal rat serum { 1: 5.....	0.5	0.4	0.3							
{ 1: 10.....				0.5	0.4	0.3	0.2	0.1	—	—
0.85 per cent NaCl.....	—	0.1	0.2	—	0.1	0.2	0.3	0.4	0.5	1.0
Inactive rabbit serum (hemolysin one unit).....	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	—
After one hour at room temperature										
Guinea-pig serum 1: 10 (complement)...	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
2 per cent suspension of rat blood-cells.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
After one hour of incubation and four hours' standing in the ice-box										
Results.....	—	—	—	=	=	+	+	++	+++	—

From table 10 we see that 0.5 cc. of ten times' dilution of the normal rat serum can almost completely inhibit the hemolytic action of just one unit of the hemolysin, when the guinea-pig serum is used as complement.

4. *Determination of a possible anticomplement substance.* Using normal guinea-pig serum (complement) instead of the one unit of hemolysin before the incubation in the above test, and adding just one unit of hemolysin and blood corpuscles afterward, we got almost the same result as in the foregoing test. The limit of the hemolysis inhibiting power of the normal rat serum against the action of the antirat hemolysin is in both cases 0.4 to 0.5 cc. of ten times the dilution of it.

By using inactive rat serum, instead of active fresh serum, we observed an equally negative hemolysis; that is, the hemolysis inhibiting substance or substances seem to be heat stable.

*c. Test of the antirat blood rabbit serum in vivo*

As a test of this antirat corpuscle rabbit serum *in vivo*, we injected a relatively large amount of this serum (0.5 cc., 1.0 cc., and 1.5 cc.) into three rats weighing 30 to 34 grams (thirty-five days of age), but observed no pathological symptoms after the injection nor any deviation of the growth curve as compared with the control animals. Fourteen days after the serum injection, all the animals were killed and examined. They were found to be normal.

From these experiments it appears that the hemolysin production in rabbits by the injection of washed rat corpuscles is, if not entirely negative, very faint. The normal guinea-pig serum can be used as complement with the antirat corpuscles hemolysin obtained from the rabbit, while the normal rat serum is quite incapable of activating the hemolysin, since it contains a substance or substances which protect the red cells in some way or other from solution by the hemolysin.

This is apparently but another case of the well-known fact that the fresh blood sera of various species differ from each other considerably in their power to activate the bactericidal or hemolytic amboceptor. In regard to hemolysis, fresh guinea-pig serum is very powerful in activating many sensitized cell complexes, but weak in activating sensitized guinea-pig corpuscles. Often we find that the alexin (complement) of an animal is entirely impotent or but slightly capable of producing hemolysis of the sensitized cells of its own species, though this is not a general rule (Zinsser, '18, p. 154). The rat complement is entirely impotent in producing hemolysis of the sensitized rat corpuscles, and this seems to result from the presence of some substance or substances in the normal rat serum, which, in some way or other, perfectly protect the rat corpuscles from hemolysis.

## 6. IMMUNIZATION OF THE CHICKEN WITH THE RED CORPUSCLES OF THE ALBINO RAT

Since I found that the combination of the albino rat and rabbit for the preparation of any strong cytolyisin (antithymus, antitestis, and hemolytic sera) is not a very favorable one, I thought it would be worth while to follow the same plan of experiment, but use some other animal than the rabbit as the serum producer.

Before using the chicken as the antithymus serum producer, we tried to get some information regarding the activity with which the chicken reacts toward the injection of the red corpuscles of rat blood. Previous to the first injection of washed red cells the normal chicken serum was examined as to its hemolytic power against rat blood, and it was found that 0.3 cc. of 1:3 dilution of it is able to hemolyze 0.5 cc. of a 2 per cent suspension of washed rat corpuscles. A 5 per cent suspension of washed red corpuscles of rat blood was used as the antigen, and 2 cc. of that suspension was injected intraperitoneally into the chicken three times, at intervals of one week.

A fresh sample of serum, taken just before the third injection, was examined for its hemolytic power. A slight positive hemolysis was obtained with 0.4 cc. of 1:10 dilution of the active immune chicken serum with 0.5 cc. of 2 per cent rat corpuscles suspension. The potency of the fresh immune chicken serum is therefore somewhat higher than the fresh normal chicken serum. Inactive immune chicken serum alone shows no hemolysis against rat corpuscles.

The same sample blood serum as before, but inactivated, shows, when tested for its hemolytic power with normal guinea-pig serum as complement, an entirely negative result. Moreover, fresh normal rat serum used with inactive chicken immune serum cannot cause any positive hemolysis.

The serum of this chicken, taken eight days after the last injection, shows negative hemolysis either with normal guinea-pig serum as complement or with normal rat serum, against washed rat corpuscles. We have to assume, therefore, if we suppose that some hemolysin production has taken place in the chicken by the injection of rat corpuscles, which seems to be very likely from

the fact that the active immune chicken serum has slightly higher hemolytic power than the normal chicken serum, that the normal guinea-pig serum and the rat serum as well, with their complements, cannot activate the hemolytic amboceptor in the chicken immune serum to produce the hemolysis against rat corpuscles.

Serologically, this is an interesting fact when we recall that some authors have used in cytolytic investigations chickens, geese, and ducks as the serum producers.

#### 7. GENERAL DISCUSSION

The attempt to get a strong antithymus serum from rabbits by the injection of rat thymus gland seems to have failed entirely. We are unable to verify Shimizu's results. His thymolysin, according to his description, must have been able to do more *in vivo* than the total removal of the thymus gland could, because we know that the surgical removal of the thymus gland can, if carefully done, be performed without any harmful effect whatever.

Moorhead ('05), reporting in a brief publication, says that his "own experiments which, however, have not been completed ('05), are so far entirely negative, although attempts have been made to immunize rabbit against guinea-pig, guinea-pigs against rabbit, and geese against guinea-pigs." He does not give any details which would explain his negative results.

We have tried to obtain a possible explanation of the negative results of the serum action and have studied further the immunization of the rabbit against rat testis and rat corpuscles. In the latter case we have learned that the hemolysin production in the rabbit by the injection of rat corpuscles is very faint, and more than that, the produced hemolysin is entirely incapable of combining with normal rat complement to hemolyze rat corpuscles—a simple example of a well-known serological fact.

If an analogy may be drawn between hemolysis and cytotoxicity of other kinds, we are tempted to explain our failure in the production of strong thymolysin when using the rabbit as a serum producer and rat thymus as the antigen, in the same way as in

the case of the hemolysin production; that is, the antibody production in the rabbit body by the injection of rat thymus is very slight, if not entirely lacking, and more than that, the normal complement of rat itself is entirely incapable of activating the antithymus amboceptor, which is produced in the rabbit's body. The same relation may be also true with regard to the anti-testis serum produced in the rabbit by the injection of rat testis emulsion. I assume, therefore, that the negative results of Moorhead's experiments may be explained in the same way; that is, the antibody production in the animal is very faint and the complement is utterly incapable of combining with the respective amboceptor to produce a true cytolysis.

I wish that Shimizu could show us that the hemolysin production in the rabbit after the injection of dog corpuscles is very active and that the hemolysin formed could combine with the normal dog complement to produce a true hemolysis. By this method of verification I think our doubt cast upon the validity of his conclusion would be greatly diminished. Following Hoskins in his discussion of Hewer's work, we may say here, "Since complete removal of the thymus has no such effects as described by Shimizu in the dogs after the injection of his thymolysin-serum, and unhealthy animals do not grow very well, Shimizu must prove that his treatment did more than injure the health of his animals."

Since we are unable to get a positive result in our experiment, and have a reasonable explanation for the negative result, we cannot believe that the thymolysin can do more *in vivo* than the total removal of the thymus could do. We are therefore inclined to the view that the retardation of the growth of the injected dogs in Shimizu's experiments, in comparison with the control, may have been caused to some extent at least by the 'primary anaphylaxis' due to the serum injection.

If Shimizu's thymolysin were not so strictly specific in reaction *in vitro* (in his paper he did not give any data for tests *in vitro*), as is the case in my antithymus serum, then his thymolysin-serum, when injected, might be expected to cause some effects other than those in the thymus gland, supposing that the complement can be combined with the amboceptors sufficiently to pro-



duce a true cytolysis in vivo. In such a case the result from the serum injection cannot be attributed to the thymolytic action only. In this connection we recall also the work of Ritchie, who obtained a serum which contained a 'leucophilic immune body,' by the immunization of ducks with the thymus gland of guinea-pigs. Further discussion regarding Ritchie's work is given later.

So long as we are unable to obtain a more solid foundation, we cannot accept the specific action of the antithymus serum, nor, furthermore, can we believe any endocrine function whatever of the thymus gland, no matter whether the cortical or medullary portion play the principle rôle in the physiological function of this gland.

So far as our results go, the chicken is not suitable as a serum producer after injection of rat material. We should not try to get the antithymus chicken serum because the hope of getting a strong antiserum is very small, and even more than that, the antithymus amboceptor produced may be incapable of causing complete cytolysis (thymolysis) in vivo, by the reason of inability of rat complement to activate the antithymus amboceptor.

We ought to recall here the report of Ritchie ('08) and a quite recent publication of Guyer and Smith ('18). The former used ducks, as already stated, for the antiserum producer and injected with the thymus of guinea-pig.

The antithymus serum obtained did not show any specific action in vivo, but fixed guinea-pig complement with the extract of thymus, lymph glands, and spleen. He concludes from his experiment that the antithymus serum from ducks contains a 'leucophilic body,' and not a specific thymolytic one; the structural changes in the thymus of guinea-pigs following injection of this serum were due to the 'leucophylic' action and were not specific. The later authors produced an antilens serum from chickens by the injection of the lens of rabbit or lens of *Peromyscus maniculatus gambeli*.

Though we have no right to cast doubt upon the validity of their conclusions, we venture to question whether the complement of guinea-pig (Ritchie) and of rabbit and *Peromyscus*

*maniculatus gambeli* (Guyer and Smith) really could activate in vivo the amboceptor which is obtained from duck or chicken by the injection of guinea-pig thymus (Ritchie) or lens of rabbit or *Peromyscus* (Guyer and Smith) and cause a cytolysis in a true sense of the word.

#### 8. RÉSUMÉ AND CONCLUSIONS

1. The serum obtained from rabbits immunized with the thymus substance of the albino rat shows some positive precipitin reaction with the thymus extract—a reaction much stronger than that from the normal rabbit serum. The specificity is, however, not strictly limited, because the testis extract shows, though slightly, a positive precipitin reaction with the antithymus serum, while kidney and spleen extracts give a little lower degree of positive reaction. Brain extract, however, does not produce any precipitation with the antithymus serum.

2. The antithymus rabbit serum does not show any positive hemolytic action against rat corpuscles when guinea-pig normal complement or rat normal serum is used as complement.

3. The antithymus sera do not cause, when injected into rats, any toxic symptoms which correspond to those of the 'primary anaphylaxis.' The injected rats do not show any marked difference in their growth from control animals. None of the organs of the injected rats show any macroscopical and microscopical alteration which might be due to the antithymus serum injection.

4. Nearly the same results were observed with the antitestis serum from rabbits injected with the rat testis emulsion. The precipitin reaction of this serum is not so strictly specific for the testis extract. This serum has no hemolytic action against rat corpuscles. All rats injected with this serum grow very well, and the testes of injected rats do not show any definite alteration caused by the antitestis serum injection.

5. Hemolysin production in rabbits by the injection of washed rat blood corpuscles was attempted. The hemolysin produced in rabbits shows a very weak positive hemolysis with normal guinea-pig serum as complement, but no hemolysis at all when normal rat serum is used as the source of the complement. That is to

say, the normal rat serum with its complement cannot activate the hemolysin against the rat corpuscles. The rat serum, even after inactivation, possesses some substance which inhibits the hemolysis. This substance may be some kind of anticomplement or of anti-amboceptor, or a combination of both of these.

6. The chicken is not suitable for the production of the hemolysin acting upon rat corpuscles with either normal guinea-pig complement or with normal rat serum as complement. Normal rat complement cannot activate (complement) the hemolysin from chicken against rat corpuscles, if anything like that substance is produced by the injection of rat corpuscles.

7. It seems very likely that our attempt to produce strong antithymus serum from the rabbit by the injection of rat thymus and to make some studies on its action have failed, partly because the antibody production in the rabbit after the injection of antigen which is obtained from the albino rat is not so active as we anticipated it would be and partly because the thymus cells of the rat are so protected from the cytolytic action of the injected antithymus serum, that the normal complement or rat serum is unable to activate the antibodies fully. The last statement is based on the analogy with the hemolysin produced in the rabbit by the injection of rat corpuscles, though the argument by analogy is admittedly dangerous.

8. From the above point of view, the publications of Shimizu, Ritchie, and of Guyer and Smith have been discussed, and I consider that their conclusion ought to have some more definite proof from the serological side. We cannot yet admit that any endocrine function of the thymus gland, either in the cortical or the medullary portion, or in both, can play the principal rôle in the physiological function of the thymus gland.

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# THE PHOTIC REACTIONS OF THE HONEY-BEE, *APIS MELLIFERA* L.<sup>1</sup>

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SEVENTEEN FIGURES

## CONTENTS

I. Introduction.....	343
II. Literature.....	344
III. Apparatus and methods.....	350
IV. Material.....	358
V. Behavior of normal bees.....	360
VI. Behavior of bees with one eye blackened.....	371
VII. Variability of photic response.....	391
VIII. Nature of photic orientation.....	403
IX. General summary and conclusions.....	410
X. Bibliography.....	412
XI. Appendix.....	415

## I. INTRODUCTION

The circus movements produced by blackening one eye in certain arthropods have long been familiar to zoölogists. It was not, however, until the advent of more recent interpretations of behavior, that they received any considerable attention. Then for the first, the significance of their relationship to normal orientation was recognized. It became apparent that the nature of the stimulus involved in the two cases was the same. Obviously, therefore, the application of any general theory of photic orientation to those forms in which circus movements occurred depended upon its ability to explain this phenomenon satisfactorily. This consideration has led, within the last few years, to a number of more or less extensive investigations of these reactions.

When the present researches were begun there had been no attempt to study circus movements quantitatively. During the

<sup>1</sup> Contributions from the Zoölogical Laboratory of the Museum of Comparative Zoölogy at Harvard College, no. 320.

progress of the experiments, however, Dolley ('16) has published a contribution to this phase of the subject. His methods as well as his results, on Vanessa, differ widely from those to be described for the honey-bee. Although in his experiments, as in mine, the illumination employed is defined as non-directive, it was very unlike in the two instances. The results obtained by Dolley are described in terms of circus movements of greater or lesser 'angles of curvature;' those obtained by the writer, in terms of degrees turned per centimeter. The conclusions drawn in the two papers are also widely divergent.

It is a pleasure here to acknowledge my deep indebtedness to Dr. G. H. Parker, at whose suggestion this research was undertaken and with whose helpful criticism it was carried on. I wish also to express my gratitude to Dr. E. L. Mark for the courtesies and privileges of the Zoölogical Laboratory.

## II. LITERATURE

As early as 1796, Goeze<sup>2</sup> (p. 42) recorded the fact that a hornet in which one eye had been painted over with an opaque varnish always flew toward the uncovered eye. Some years later, Treviranus ('32, p. 194) described an experiment in which the lower half of the right cornea of a dragon-fly was carefully cut away from the optic nerve, with the result that the animal moved toward the left side.

Decidedly the most interesting of the earlier observations are those of Dubois ('86) on a phosphorescent elaterid beetle of the genus *Pyrophorus*. This insect responds positively to at least certain intensities of light, and according to Dubois (p. 209) it is most affected by the yellow-green rays, which also predominate in the spectrum of its own light. The photogenic organs are three in number, one occupying a median ventral position on the first abdominal segment, the other two being situated on opposite sides of the prothorax near its dorsolateral edges. Whenever the beetle begins to creep spontaneously in the dark, the prothoracic organs become luminous. During flight the abdominal organ does likewise.

<sup>2</sup> I have not had direct access to this work. The above reference is taken from a footnote in Treviranus ('32, p. 193).

Dubois (p. 208) found that upon completely obscuring the light from the prothoracic organ of one side of the body with a covering of black wax, the beetle no longer crept in a straight line. Smoked paper records, made in a dark room, showed that such individuals crept in circles toward the functional eye. A check experiment, moreover, showed that the results obtained were not due to the weight of the wax. If instead of eliminating one of the prothoracic organs, the cornea or the entire eye of one side was destroyed with a red-hot needle (p. 211), very similar results were obtained. When, however, both photogenic organs of the prothorax were obscured or both eyes were destroyed, the animal crept in a hesitant, irregular fashion, presently stopping altogether.

Dubois has interpreted these results from an anthropomorphic viewpoint, as evidenced by his original paper and by a more recent comment ('09). To the present writer, however, these responses of *Pyrophorus* afford not only a typical case of circus movements, but one of considerable theoretical importance as well. The tendency to circle attendant upon the suppression of one photogenic organ or the destruction of one eye may be attributed to the unequal stimulation on the two sides of the body. If this be correct, the case is indeed unique, for the beetle is oriented by its own luminosity. This, of course, in nowise affects behavior in the normal animal. With a photogenic organ on each side of the prothorax producing light of the same quality and intensity, it is always perfectly oriented with respect to its own light. But if the source of light or the photoreceptor of one side be eliminated, the beetle promptly orients toward the opposite side, the side which is receiving the greater stimulation.

In recent years, a steadily increasing number of arthropods have been shown to exhibit circus movements when one eye is blackened or destroyed. The researches of Bethe ('97 a), Axenfeld ('99), Holmes ('01, '05), Rádl ('01, '03), Parker ('03), Hadley ('08), Carpenter ('08), Brundin ('13), Holmes and McGraw ('13), Dolley ('16), and Garrey ('17), have demonstrated conclusively that among phototropic arthropods generally, unilateral photic stimulation results in a more or less asymmetric response.

These investigations have covered between fifty and sixty species, including the four chief classes of arthropods. Among the insects, where most of the work has been done, representatives of most of the larger orders have been experimented upon. These embrace Orthoptera, Blattoidea, Hymenoptera, Coleoptera, Odonata, Lepidoptera, Diptera, Homoptera, and Hemiptera. The phenomenon of circus movements—or perhaps better, asymmetrical response—must, therefore, be regarded as general rather than exceptional for the members of this phylum.

The form of response naturally varies with the peculiarities of locomotion in a given species. It is not the same for a sidewise moving crab, such as *Carcinus*, as it is for an insect which moves forward. With the usual type of forward locomotion, however, arthropods with one eye blackened generally circle toward the functional eye, if they are positively phototropic; toward the non-functional eye, if they are negatively phototropic.

It is true there are cases which, on first examination, do not appear to conform to this generalization. Thus Holmes ('05, pp. 332–336) has demonstrated clearly that an animal with one eye blackened may at first perform circus movements in creeping toward a light, only to modify its behavior after a time and creep in a straight path. Such was true of both *Ranatra* and *Notonecta*. Axenfeld ('99, p. 375) had previously made similar observations, and more recently Brundin ('13, pp. 337, 346–348) and Dolley ('16, pp. 371–382) have demonstrated the same phenomenon in the species with which they worked.

There can be no doubt, therefore, that many arthropods with one eye blackened are able in time to modify their behavior to light. This, however, in no wise lessens the significance of the initial tendency of the animal to perform circus movements. In fact, this initial tendency is the all-important one as far as the question of orientation in the normal animal is concerned. I do not believe, therefore, that the presence of modifiability in an animal warrants considering its behavior as an exception to the general occurrence of circus movements.

A second difficulty in the way of any generalization concerning circus movements has been encountered in the behavior of cer-

tain flies. Thus Rádl ('03, p. 62) says, "Die *Calliphora vomitoria* bewegt sich fast ebenso gerade mit einem geschwärzten Auge, wie wenn sie aus beiden sieht, und es ist mir nicht leicht, diese Erscheinung zu erklären." Carpenter ('08, p. 486) states that *Drosophila* with one eye blackened "crept in a fairly direct path toward the light, although a tendency to deviate toward the side of the normal eye regularly occurred." It is possible, I believe, to interpret these cases as merely more extreme instances of modifiability, in which regulation occurs very rapidly instead of after a more or less prolonged experience.

That modifiability is operative, at least in the case of *Drosophila*, is evidenced by the following statement of Carpenter (p. 486). "The tendency to diverge from the direct path toward the side of the uncovered eye was overcome by a series of short, quick turns in the opposite direction, which kept them headed toward the light." Further evidence in the case is afforded by the behavior of one fly which, according to Carpenter, persisted in performing circus movements. This fly, however, (p. 486) "had long been active, and showed signs of fatigue." As will be shown later, very similar phenomena were observed in the honey-bee. In conditions, such as that of weakness, induced by long experiment, the bee frequently circled much more toward the functional eye than it had formerly done. It seems probable that in such states the animal approximates more nearly to a simple, reflex behavior. Factors effective in modifying behavior in the vigorous animal have ceased to be operative.

If these interpretations be correct, the conspicuous absence of circus movements in *Drosophila* is only an extreme case of modifiability, and offers no real objection to the general conclusion to be drawn from these reactions. However, further work is necessary upon both *Drosophila* and *Calliphora* before they may be disposed of with certainty.

Responses of still another kind have seemed perhaps the most formidable obstacle to any general conclusion as to the occurrence of circus movements. Thus Hadley ('08, p. 197) has shown that whereas the 'progressive orientation' of the lobster larva after the blinding of one eye is positive, the larva performs circus



movements or turns toward the injured side. Brundin ('13, p. 346) states that in positive specimens of *Orchestia traskiana*, circus movements will occur as often toward the blackened as toward the normal eye, while Holmes and McGraw ('13, p. 370) report the case of a positive skipper butterfly which almost invariably circled toward the blackened eye.

A very plausible explanation of these apparent anomalies, however, has been offered by Dolley ('16, pp. 394-399), who has shown that the contact stimulus afforded by the material covering the eye is sufficient to cause Vanessa, when in the dark, to turn continuously toward the covered eye. This tendency, moreover, exhibits little, if any, modification from day to day. The effect of such a contact stimulus is continuous. But in the presence of photic stimulation of moderate or high intensity, it is quite overwhelmed by the strong phototropism of the butterfly. In the case of animals of less certain phototropic index, this contact stimulus is, in all probability, frequently strong enough to overcome the effect of light. An examination of the cases cited above shows that the phototropism of these animals is not of the unequivocal kind exhibited by Vanessa. It seems likely, therefore, that their apparently exceptional behavior was due to contact and not to photic stimulation.

Suppressions of photic circus movements by responses to other stimuli are not surprising, when it is recalled with what facility even the stereotyped circus movements produced through unilateral lesions of the central nervous system may be altered in a similar manner. Thus Bethe ('97 b, p. 507) states that the tendency of bees to circle toward the normal side after the removal of one half of the brain or the severance of one of the oesophageal commissures, may be arrested, and the animal may even be compelled to deviate toward the injured side by stimulating the legs of the normal side. Moreover, in a general statement concerning the several crustaceans and insects subjected to similar operations (p. 541), he says, ". . . nach Aufhebung der Hemmung der gesunden Seite durch angebrachte Reize aber auch spontan bei allen Versuchsthieren gerader Gang und Kreisgang nach der operirten Seite eintritt."

Whether the effect of contact stimulation also accounts for certain of the phenomena observed by Axenfeld ('99) is not so clear. Axenfeld reports that nocturnal lepidoptera with one eye blackened turned toward the blackened eye during the day. In the same paper he makes the following general statement: "Enfin on peut observer que ces mêmes animaux photofuges, qui tournent en pleine lumière du soleil du côté de l'oeil couvert, offrent le mouvement contraire au soir ou même de jour, quand ils sont transportés dans une chambre mal éclairée; . . . ." It may be that such animals, being attuned to a low intensity, respond positively to it, whereas a stronger intensity evokes a negative reaction, somewhat according to the idea of Davenport ('97, p. 197). Certainly, if the circling of the nocturnal lepidoptera toward the covered eye was a light response, it is not in harmony with the statement of Loeb ('90, p. 51) to the effect that all 'day and night butterflies' are without exception positively phototropic. I am led to suspect, however, that some of the reactions noted by Axenfeld were the results of contact stimulus, for Hess ('13 a, p. 651) has shown that *Coccinella*, which Axenfeld reports as circling toward the blackened eye, is not negative to light. Axenfeld's experiments, therefore, need careful repetition before any final conclusions may be drawn from them.

It seems quite certain, therefore, that what have appeared to be exceptions to the general occurrence of circus movements among phototropic arthropods are not really incompatible with this view. Taken as a whole, the investigations of these reactions demonstrate rather conclusively that, although they may be modified through experience or obscured by responses to other than photic stimuli, they are, nevertheless, to be considered as characteristic of phototropic arthropods. Photic orientation in this group of animals, therefore, cannot be accounted for by any theory which fails to offer a satisfactory explanation of circus movements.

## III. APPARATUS AND METHODS

1. *Directive Light*

In the experiments of the present paper, both directive and non-directive light were employed. Those involving directive illumination were carried on in a circular area (fig. 1) 2.44 m. in diameter, which was laid out in black lines on the concrete floor of a dark room. Sixteen centimeters<sup>3</sup> above the center of this area, an incandescent lamp was suspended. The lamp employed was a 100-watt, 115-volt, stereopticon, Edison mazda lamp. Of several bulbs used in the course of experimentation, only the last was determined photometrically, its candle-power being approximately 80. These lamps when new are calculated to furnish 100 c.p., but their efficiency decreases considerably with usage.

In making tests in the directive light area, bees were started creeping at the outer circumference. The course of the animal as it traveled toward the light was then traced as accurately as possible on a record bearing a plan similar to that of the light area and drawn to scale. Such a record is shown in figure 1.

2. *Non-directive light*

*a. Construction.* The apparatus employed to furnish non-directive light consisted essentially of a white-walled, cylindrical chamber. This chamber was illuminated by an incandescent lamp, the light of which was diffused through a thin, white screen, suspended a short distance below the lamp. Bees were admitted to the apparatus through a small, circular opening in the center of the floor, and the course of their creeping was then traced as accurately as possible on a record. The apparatus was especially designed to afford a creeping animal a continuous photic stimulation of uniform intensity over the entire surface of the eye. A more detailed description is presented in the following paragraphs (see figure 2).

<sup>3</sup> Distances from lamps to creeping surfaces were measured from the center of the filament in all cases.

The cylindrical chamber, which measured approximately 84 cm. in height by 87 cm. in diameter, was constructed on a light wooden framework covered on the exterior with heavy, corrugated cardboard. On the interior it was lined with a thickness

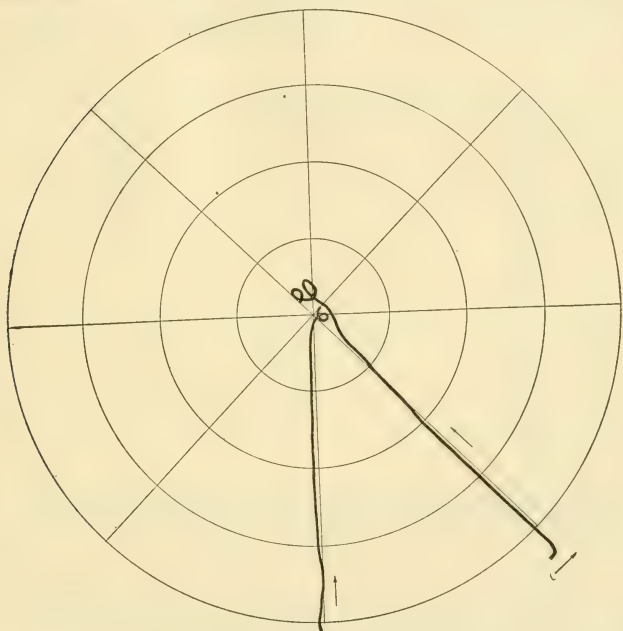


Fig. 1 Plan of directive light area, showing two trails of a normal bee. Note the deflection of the courses in the non-directive region near the lamp and directly beneath it.

of dead white, cotton cloth, backed by a layer of heavy white paper. On one side of the cylinder, and extending from its bottom edge, a rectangular opening 58 cm. high by 32 cm. wide was cut through the cardboard and paper layers. The white cloth lining only closed this opening, and it was here slit from top to bottom, the bottom edges being left free. The two flaps thus

formed allowed free access to the interior of the cylinder. In one of them a small opening (fig. 2, *o*), 3 by 4 cm., was cut for purposes of observation.

The top of the cylinder was similar in construction to the side walls except that the cardboard layer was omitted. Near opposite edges of the top, two circular openings, 8 cm. in diameter,

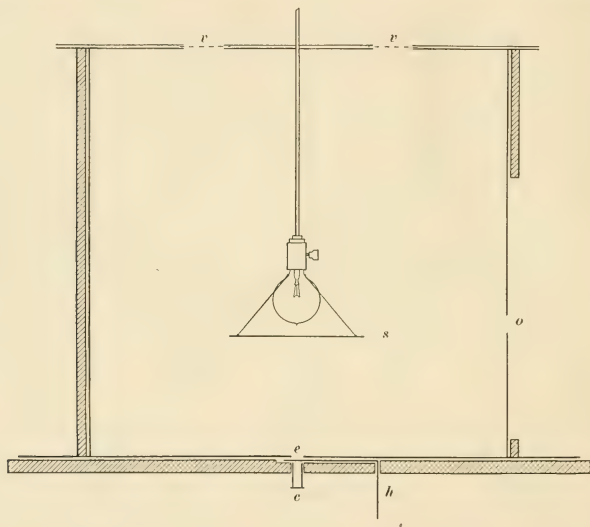


Fig. 2 Diagrammatic section through non-directive light apparatus. *c*, transferring cage; *e*, entrance to light chamber; *h*, handle of slide opening and closing *e*; *o*, opening for observation; *s*, light screen; *v*, ventilators.

were cut (fig. 2, *v*). These were covered with a thin, white gauze of coarse mesh, and served as ventilators, preventing any undue rise of temperature within the apparatus. The bottom of the cylinder was formed by a layer of heavy, dead white paper, which covered the table on which the cylinder stood. This paper was especially selected to afford a good creeping surface. On it was drawn a plan, similar to that shown in figure 3, by means of which the course of a creeping bee could be accurately followed.



The illumination of the apparatus resembled the semi-indirect illumination of a modern house, the light from an incandescent lamp being diffused through a circular screen (fig. 2, *s*), 22 cm. in diameter, of white bond paper. Two intensities of illumination were employed. The less intense was produced by a carbon filament lamp of approximately 2 c.p.,<sup>4</sup> 66 cm. above the floor, and the more intense by the 80 c.p. mazda lamp previously described, 33 cm. above the floor. The intensity of illumination in each instance was measured at three different points on the floor of the cylinder. One determination was made at the center; a second at a point 3 cm. from the right side wall, and a third, at a point 3 cm. from the left side wall. The results of these measurements are given in table 1. Hereafter, in referring to the

TABLE 1

A	B	C	D	E	F
CANDLE-POWER OF LAMP	INTENSITY ON FLOOR AT CENTER	INTENSITY ON FLOOR 3 CM. FROM RIGHT SIDE WALL	INTENSITY ON FLOOR 3 CM. FROM LEFT SIDE WALL	AVERAGE OF C AND D	AVERAGE OF B AND E
<i>c.p.</i>	<i>mc.</i> <sup>5</sup>	<i>mc.</i>	<i>mc.</i>	<i>mc.</i>	<i>mc.</i>
2.36	25.9	17.93	25.25	21.59	23.75
79.45	1051.5	831.37	894.45	862.91	957.21

two intensities of illumination employed, the averages given in the table will be used in round numbers. The less intense will be designated as non-directive light of 24 mc.; the more intense, as non-directive light of 957 mc.

The transference of bees to and from the apparatus was effected by means of a small, cylindrical cage of wire screen, 5 cm. in length by 2 cm. in diameter (fig. 2, *c*). This cage, one end of which was open, exactly fitted into a circular opening cut through the table top to the center of the chamber floor. By means of a

<sup>4</sup> The lamp used throughout experimentation was, unfortunately, broken before being determined photometrically. Its candle-power was certainly between 2 and 4.

<sup>5</sup> Throughout the present paper, the abbreviation mc. will be used to designate meter candles.

slide operated by a handle (fig. 2, *h*), it was possible, after inserting the cage, to open or close the light chamber at will. The difficulties involved in direct manipulation of bees were thus entirely avoided. An individual to be tested in non-directive light was merely allowed to creep into the cage, which was then inserted into the opening in the table top. The slide was then pushed aside and the bee allowed to creep up on to the floor of the apparatus. As soon as the bee had entered the light chamber, the slide was pushed back, closing the entrance and leaving the floor of the apparatus complete.

The ideal apparatus for studying the effects of continuous photic stimulation of a constant intensity would be one so constructed that all the ommatidia of a compound eye would receive equal illumination, irrespective of the direction of locomotion. Such an apparatus is virtually a physical impossibility. However, the apparatus just described is perhaps somewhat of an approximation to it, even if it does not afford an absolutely uniform light intensity over the floor of the light chamber. As table 1 shows, the illumination is more intense toward the center. Some fluctuation will, therefore, occur in the stimulation of the various ommatidia as the animal moves. However, in any position whatever on the floor of such a light chamber, all the ommatidia are receiving some stimulation. Moreover, the amount of stimulation received by those areas of the eye which are minimally affected does not differ vastly from that received by areas of maximal stimulation.

*b. Records.* The method of recording behavior in non-directive light is illustrated in figures 3 and 4. The animal to be tested was transferred to the light chamber, and its course of creeping, observed through the 'peep hole' in the curtain, was traced as carefully as possible on a record sheet. The record bore a plan similar to that on the floor of the light chamber, drawn on a scale of 1 to 6. The duration of each trial was ascertained by counting the rings of an electric bell, attached to an electric clock regulated to seconds. How long the trial should last was determined by an interval previously decided upon or by the animal encountering the side wall of the chamber and creeping up. On completion of

a trial, the bee was removed, and the remaining data called for on each record were entered. The tracing was marked with arrows to indicate its direction. Observations on the physical condition of the animal and others of importance, which were made from time to time, were also noted on the record. All the records of a single animal were then filed away together, thus affording a permanent record for further reference.

Since it was desired to make a quantitative study of circus movements, it was necessary to adopt some method whereby the amount of turning exhibited by an animal, in a given trial or group of trials, might be expressed as a single value. These values have been stated in terms of average number of degrees turned per centimeter of progress, and were obtained in the following manner. The length of the trail was first measured with a map tracer. Several readings were taken until two were obtained with a difference of less than 0.3 cm. These were then averaged, and the result used in computations. Thus in figure 3, the length of the original tracing is 26.95 cm. Since, however, the records were on a scale of 1 to 6, and these in reproduction have been reduced one half, the length of the text figure tracing must be multiplied by ( $6 \times 2 =$ ) 12 in order to obtain the distance actually traveled by the animal.

The various turns or angular deflections of the trail were next estimated by reference to the radii of the plan. It is obvious that in traveling a curved course, the direction of locomotion at any given instant is the tangent to the curve at that point. For example, in figure 3 the initial direction of locomotion is shown by the tangent at *a*. This direction is parallel to a radius. From *a*, the tangent to the curve or the direction of locomotion rotates continuously to the left until the point *b* is reached. At *b* the tangent is parallel to a second radius, which makes with the radius of initial parallelism an angle of  $\frac{1}{3}$  of  $360^\circ$  or  $180^\circ$ . (Each radius forms angles of  $45^\circ$  or  $\frac{1}{8}$  of  $360^\circ$  with its adjacent radii.) In other words, in traveling from *a* to *b* the axis of the animal's body has rotated  $180^\circ$  to the left, or the animal has executed  $\frac{1}{2}$  of a complete sinistral loop. Similarly, from *b* to *c* the course of the animal makes  $1\frac{3}{4}$  dextral loops; from *c* to *d*,  $\frac{1}{8}$  of a sinistral

loop, and finally from *d* to *e*,  $1\frac{1}{8}$  dextral loops. The total amount of turning, or angular deflection, toward the right in this trail is, therefore,  $1\frac{3}{4} + 1\frac{1}{8}$  or  $2\frac{7}{8} \times 360^\circ$ , while that to the left is  $\frac{1}{2} + \frac{1}{8}$  or  $\frac{5}{8} \times 360^\circ$ .

Since the honey-bee is positively phototropic and in this case the left eye was blackened, the angular deflection toward the right or functional eye is designated as positive; that toward the left or covered eye, as negative. The algebraic sum of these

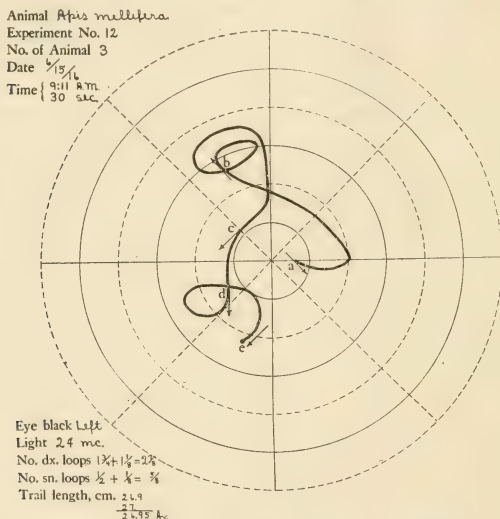


Fig. 3 Record of bee no. 123 in non-directive light.

angular deflections will give a result equivalent to the amount of continuous turning required to carry the animal from the starting point to the end of its course. Thus, in figure 3, the direction of locomotion at *a* makes with the direction at *e* an angle of  $2\frac{7}{8} \times 360^\circ - \frac{5}{8} \times 360^\circ$  or  $2\frac{1}{4} \times 360^\circ$ .

Knowing the distance traveled in centimeters and the amount of turning in degrees, the average degrees turned per centimeter is easily computed. Denoting this average deflection, as I shall call it, by *D*, we have for the trail in figure 3,

$$D = \frac{2\frac{1}{4} \times 360^\circ}{26.95 \text{ cm.} \times 6} = +5.01^\circ/\text{cm.}$$

It is to be emphasized that the value  $+5.01^\circ/\text{cm.}$ , does not signify that the animal turned only toward the functional eye. It merely shows that the algebraic sum of all its deflections averages  $5.01^\circ/\text{cm.}$  toward the functional eye.

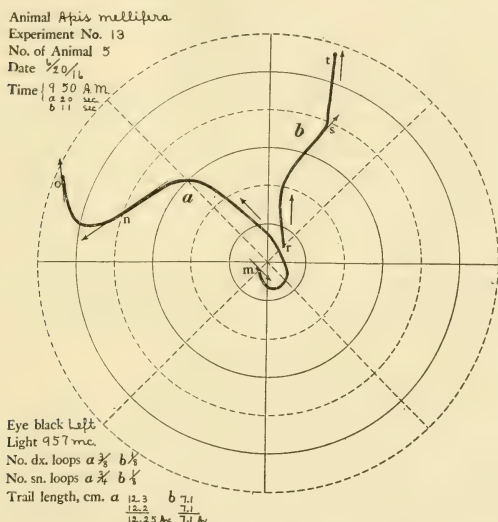


Fig. 4 Two trails of bee no. 135 in non-directive light.

A record is shown in figure 4 which represents two trials taken in rapid succession. This was necessitated by the animal's encountering the side wall of the light chamber so quickly that the first trial was shorter than usual. The deflections in these trails are estimated as previously described. It will be noted, however, that in the trail marked *a*, the angular deflection between *m* and *n* does not amount to quite  $\frac{3}{4}$  of a sinistral loop, although it is so counted. In such instances the angle was always estimated



to the nearer  $\frac{1}{8}$  of a circumference, no attempt being made to discriminate differences of less than  $45^\circ$ . In this record it is desirable to combine both trails into a single computation. Proceeding as before,

$$D = \frac{(+\frac{3}{8} + \frac{1}{8} - \frac{3}{4} - \frac{1}{8}) 360^\circ}{(12.25 + 7.1 \text{ cm.}) 6} = -1.16^\circ/\text{cm.}$$

The negative sign of the average deflection here obviously indicates that the bee turned more toward the covered eye than toward the functional eye in these trials.

In the course of experimentation, records of normal bees were also made in non-directive light. Since in such individuals neither eye was blackened, the positive sign was arbitrarily applied to the direction of greater angular deflection in each set of trials. Otherwise the computations for normal bees were performed in the same manner as those for bees with one eye blackened.

These various examples will illustrate the method employed in all quantitative determinations. Upon the results thus obtained the chief conclusions of the present paper are based.

#### IV. MATERIAL

##### 1. *General care of animals*

The bees used in all quantitative experiments were thoroughly active workers taken from the flowers of a near-by garden, and were, for the most part, individuals from a single large hive situated there. The animals were trapped by simply inverting a long glass tumbler over the flower, and then transferring them to a small screen fly-trap. In some experiments, however, which were performed too late in the fall to obtain bees in this way, animals were used from a single comb of workers confined in an observation hive. The exit of the hive was kept securely screened, for such a colony quickly disintegrates if its members are permitted to leave the hive freely. Bees kept in this way remained in reasonably good condition, for at least a month.

Bees destined to undergo experimentation were first subjected to having their wings clipped, an operation easily executed when

the animals were feeding. Each wingless individual was then confined in a small cylindrical cage of screen wire, the bottom of which was formed by a layer of tissue-paper over cotton to prevent injury in case of falling. In the same cage were also placed two friendly winged workers to counteract any possible effects of isolation. The cages of bees were kept in a darkened box when not directly under experimentation, since the influence of light often caused the animals to maintain a restless activity which appeared, in some cases, to shorten life considerably. In the dark, however, they usually remained more quiet.

Each cage was supplied with water by a small wad of saturated cotton placed on its top. Small quantities of honey were also supplied on short wooden sticks stuck to the side of the cage. Early in the morning, at noon, and in the evening the cages were cleaned by removing excess honey, etc., and fresh honey and water were provided. Such operations were carried out at least a half-hour before any trials were made on the animals.

The temperature of the laboratory in most of the experiments was kept above 20° to 21°C. This was found to be an important consideration, since at lower temperatures bees became torpid and inactive. In collecting the animals even, an attempt was made to take them on warm, sunny days which had, in general, been preceded by warm weather. It was found that bees brought in after a brief period of cold, wet weather were apt to be either unresponsive or extremely variable in their behavior.

## *2. Blackening the eye*

Any technic for blackening the eye of a wingless bee requires, of course, the use of an anaesthetic. In the present experiments ether was used exclusively. Care was taken to administer it rather slowly and in minimal doses. When completely anaesthetized, the bee was placed on one side, on a small cork pinning board. Here it was fastened down securely by the use of insect pins, with which the thorax, abdomen, and legs were securely braced against the cork. The blackening was then applied to the eye, the entire surface being covered with as thick a coat as pos-

sible. Two kinds of blackening material were used, viz., lamp-black in shellac and a dead black paint known commercially as 'Jap-a-lac.' The latter proved the more satisfactory and was used throughout the majority of experiments.

Although bees under ether often began to recover in five to ten minutes, they were not removed from the pinning board for twenty to twenty-five minutes, when the covering of the eye was well hardened. Recovery from anaesthesia was usually complete in an hour and often much less. As a rule, however, operations were carried out in the evening, and the bees were not subjected to further experiment until the following day. Ample time was thus allowed for the animals to recover as much as possible from the effects of the operation.

## V. BEHAVIOR OF NORMAL BEES

### 1. *Kinetic effect of light*

The remarkable sensitivity of the honey-bee to photic stimulation must have long been patent to students of its behavior. Bethe ('98, p. 83) says, "Das Licht ist bei diesen Tagthieren [bees, flies, etc.] der auslösende Reiz zum Fliegen; in einer dunklen Schachtel fliegt keine Biene auf, auch nicht, wenn man sie reizt. Das Licht gibt die Regulirung beim Fluge ab." This observation was repeatedly confirmed in the present experiments. When collecting bees from flowers, fifteen to twenty individuals were confined in a single cage, which was then placed in a closed box. Although at the height of activity when captured, a few minutes in the darkness of the box seldom failed to reduce these animals to a state of quiescence. If a little light was admitted to the box, however, by even partially removing the lid, there was a sudden resumption of activity.

Precisely the same behavior was exhibited by wingless bees. If confined in a dark box, they were, as a rule, reduced to comparative inactivity. A brief exposure to light, however, was usually sufficient to excite vigorous locomotion, and continued exposure not infrequently resulted in the maintenance of an intense activity for extended intervals of time.

Individuals which had been subjected to operations of removing the wings and blackening the eye frequently responded somewhat more slowly to this photic activation than did normal bees. In the former, locomotion was preceded by a more or less prolonged sequence of cleaning operations. The proboscis was extended and stroked with the fore legs. The eyes, particularly the covered one, were the objects of repeated and vigorous scrapings, responses no doubt largely attributable to the irritation of the blackening material. The abdomen was meanwhile bent from side to side, while the middle or hind legs were rubbed together, or the hind legs assiduously stroked the dorsum of the abdomen. These movements became more and more intense until at length they culminated in active creeping.

Light, then, exerts a strong activating or kinetic influence upon the honey-bee, while darkness has the opposite effect. Essentially similar phenomena have been reported by Loeb ('90) for the plant louse, Carpenter ('05) for the pomace fly, and Turner ('12) for the mason wasp. Stockard ('08) has reported the case of *Aplopus*, the 'walking-stick,' which also falls into this category of behavior. In *Aplopus*, however, light inhibits activity, while darkness induces it. Hence the 'walking-stick' is nocturnal, whereas the plant louse, the pomace fly, the mason wasp, and the hive bee are diurnal.

In diurnal animals this response is apparently due to the continued action of light rather than a sudden change in it. Thus, while many bees respond almost, if not quite, at once to the presence of light, others may respond only after some minutes of exposure. According to Turner ('12, p. 360), the same is true of the mason wasp.

## *2. Directive light*

Not only does light induce locomotion in the honey-bee, but directive light regulates the course of locomotion. Bees brought into the laboratory direct from their foraging activities out of doors seldom failed to exhibit a most striking phototropism. Such insects when liberated in the laboratory flew almost immediately to the nearest window, where they remained fluttering

against the glass. Or, if escaping in a darkened room, they not infrequently flew directly into the flame of the nearest gas jet.

Observations of this sort were long ago reported by Lubbock ('82, pp. 278, 279, 284). A few years later, Graber ('84) demonstrated the same thing experimentally by confining forty to sixty bees in a small box, one half of which was illuminated by direct sunlight, the other half being shaded, with the result that the majority of the bees soon collected in the illuminated end. More recently, Hess ('13 a, '13 b, '17) has repeated this and a variety of other experiments. As a result of these he has been able to show that in the presence of several sources of photic stimulation, which differ in color and intensity, bees always orient toward the one which to a totally color-blind person appears brightest. The positive phototropism of the honey-bee is thus demonstrable in a variety of ways.

In the experiments just cited, winged bees were used exclusively. My own experiments, on the contrary, were confined entirely to workers from which the wings had been clipped. Such bees when creeping in the directive light area exhibited an orientation which was striking in three respects, viz., its rapidity, its precision, and its constancy.

An individual to be tested was removed from the dark box and exposed to light for a few minutes until it was thoroughly active. It was then allowed to creep from its screen cage to a small, rectangular piece of black paper, and on this it was transferred to the edge of the directive light area. An effort was made to start the animal creeping at a right angle to the direction of the light rays by turning the paper just before it crept off. The rapidity of orientation was so great, however, that the intervening centimeter or so was frequently sufficient to allow the animal to reorient perfectly. Since the velocity at which bees creep averages 3 to 6 cm. per second, orientation in these cases occurred in considerably less than one second. I have also tried leading a bee by moving the light, now in this direction, now in that, with varying degrees of curvature. Always the animal followed, orienting rapidly to even slight movements of the lamp.



The precision with which orientation was maintained was no less conspicuous. Once oriented, the animal generally moved in a nearly straight line toward the source of light. In figure 5 are shown two records of each of six bees in the directive light area. Of a large number of animals tested in the course of experimentation, considerably over 25 per cent maintained their orientation as precisely as did bee no. 66. The deviations of

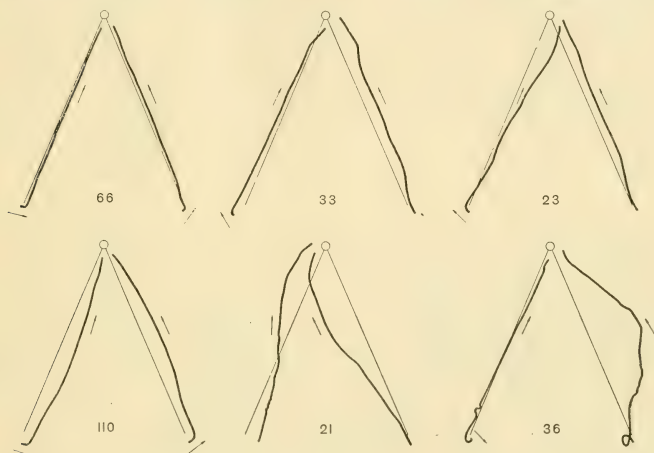


Fig. 5 Two trails of each of six normal bees in directive light. In this, as in subsequent figures of records in directive light, the clear circle represents the light source, and the straight lines from it, the direction of the rays.

most of the animals would, moreover, easily fall within the latitude of that exhibited by bees nos. 66, 33, and 23. Results similar to those shown for animals nos. 110 and 21 were, on the contrary, less frequently encountered, while trails such as those of bee no. 36 were seldom or never found among normal, healthy bees.

The response to directive light is very constant in the bee. The oncoming of death itself seems often to intensify rather than to weaken this phase of its behavior. Bees occasionally escaped

in the laboratory. Such individuals rarely survived the lack of food for more than a day or so. Yet it was not an infrequent occurrence to observe one of these starved animals, so weak that it was barely able to creep, slowly emerging from a hidden corner in a final struggle toward the light.

Nevertheless, bees were discovered which in a few instances failed to exhibit the usual positive reaction to directive light. Such cases, however, are not to be construed as a total absence

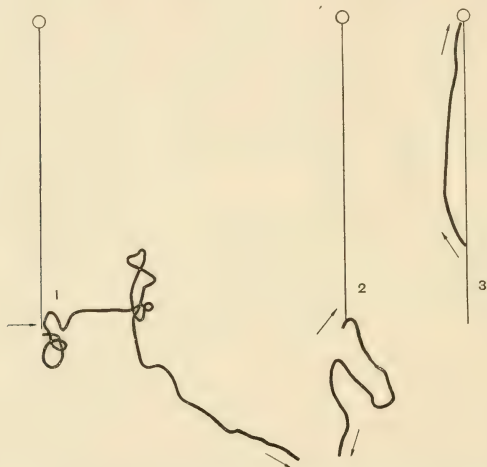


Fig. 6 Three successive records of a normal bee in directive light, showing a failure to orient in two cases.

of phototropism, but rather its momentary suppression by other factors of behavior. This is well illustrated by the following example. Seven cages of bees were prepared from the stock in the observation hive, Oct. 30, at 2:45 P.M. When tested about an hour later in the directive light area, six of the seven animals exhibited the usual positive response. One animal, however, gave the records reproduced in figure 6.

This bee when given its first trial at 4:06 (fig. 6, 1) did not orient toward the light source. Instead it pursued a devious course

looping now to the right, now to the left, and finally turning almost directly away from the light. In a second trial at 4:14, it exhibited a somewhat similar response (fig. 6, 2). One minute later, the animal was subjected to still a third trial, being started on this occasion some 30 cm. nearer the light. This time it oriented and moved in a fairly direct course toward the source of illumination (fig. 6, 3). What the temporary, inhibiting factors were which produced these very atypical responses could not be ascertained. In all other respects this bee was quite indistinguishable from the other individuals in the experiment. This example, however, shows that even the constant response of the bee to directive illumination is not free from abrupt and apparently inexplicable departures.

### *3. Non-directive light*

The behavior of bees in non-directive light is no less characteristic than that in directive illumination. Since all quantitative experiments on circus movements were conducted in non-directive light, an intimate acquaintance with the behavior of normal animals under the same conditions was necessary. Every bee was, therefore, subjected to several trials in non-directive light before having one eye blackened.

It was a matter of continual observation that a bee creeping in the directive light area ceased to move in a straight course upon reaching the area near and immediately beneath the lamp. Here, where the illumination was essentially non-directive, the animal deflected from its former, precise path and began to loop in a constant or varying direction (fig. 1). In other words, the bee was trapped; for directly it crept away sufficiently for the light to become directive again, it was forced to turn back. Thus the animal continued to creep round and round in a limited area, occasionally rearing on its hind legs in an abortive attempt at flight, or finally ceasing locomotion to begin cleaning operations.

In the non-directive light apparatus (fig. 2), the same tendency to loop was manifested, only on a much more extensive scale. Here the bee seldom crept in a straight line for any great

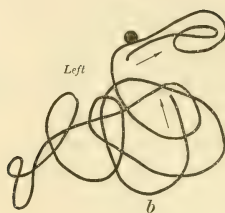
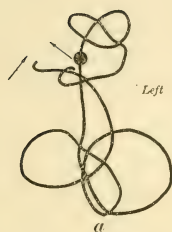
distance. Each animal was subjected to two sets of trials, an hour or so apart. Usually a single trial only constituted a set. In case the bee quickly encountered the side wall of the light chamber, however, or exhibited unusual variability in its behavior, additional trials were made. The aggregate duration of the trials of each set varied considerably, even in the same animal. Sometimes they were as short as thirty seconds; again, as long as two minutes. The average was in the neighborhood of thirty to sixty seconds. Preliminary to each trial, the bee was exposed to light until aroused to active creeping. The illumination used throughout in experimenting with normal bees was 957 mc.<sup>5</sup>

The average deflection to the right or to the left has been computed for each set of records thus obtained, and the results of these computations presented in table 2 (appendix), columns B and C. On the basis of these data, the fifty-two bees experimented upon may be classified into three groups:

1. Bees whose average deflection in both sets of trials was over  $2^{\circ}$ /cm. and in the same direction.
2. Bees whose average deflection in both sets of trials was small.
3. Bees whose average deflection in the two sets of trials varied widely, either in magnitude or direction, or in both.

The first class is composed of animals which exhibited a more or less pronounced tendency to turn in a constant direction (right or left). These animals, 29 in number, comprised 56 per cent of the total 52 bees. Fourteen of these were chiefly right-handed in their turns; 15, left-handed. In 14 of the 29 bees, or 27 per cent of the total number, the average deflections exceeded  $4^{\circ}$ /cm., while in 6 individuals, or 12 per cent, it rose to over  $8^{\circ}$ /cm. Similar right and left-handed tendencies of locomotion in non-directive light have been reported by Walter ('07) for planarians and by Patten ('14) for the blowfly larva. A typical example of this behavior in bees is illustrated in figure 7, bee no. 101. In its first trial (fig. 7, 101, *a*), this animal showed an average deflection of  $7.11^{\circ}$ /cm. to the left, and in the second trial (fig. 7, 101, *b*), a similar deflection of  $6.10^{\circ}$ /cm. Since these records were made nearly an hour apart the left-handed tendency was not the result of a

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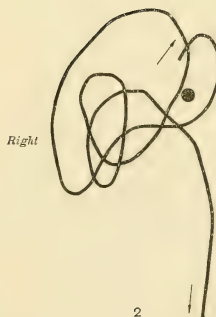
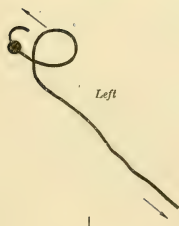
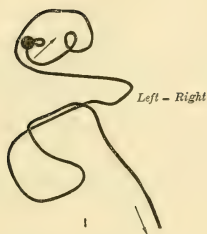


Fig. 7 Records of normal bees in non-directional light. In this, as in subsequent figures of records in non-directional light, a solid circle is used to indicate the center of the floor of the non-directional light apparatus. *a*, records of the first set of trials; *b*, records of the second set of trials. Bee no. 101 deflected constantly toward the left. Bee no. 36 varied its deflection in the course of single trials.



brief, temporary condition, but was probably a more or less permanent feature of this animal's behavior.

The second class of animals includes those whose average deflections were small in both directions. The results obtained here are attributable to either of two causes:

*a.* The bee varied its turning from right to left, so that on an average, one tendency nearly or quite balanced the other.

*b.* The bee exhibited little or no tendency to turn either to the right or to the left.

An example of the first type is seen in the records of bee no. 36, figure 7. In its first set of trials (*a*, 1, 2) this animal turned sometimes to the left, sometimes to the right, so that the resultant average deflection was but  $0.79^{\circ}$ /cm. to the left. Similarly in the second set of trails (*b*, 1, 2), the average deflection amounted to only  $1.94^{\circ}$ /cm. to the right. The second type of this class is illustrated by bee no. 134, figure 8. This animal showed no pronounced tendency to turn either to the right or left. The average deflection for each set of records was, therefore, small, being only  $1.52^{\circ}$ /cm. to the left for the first set (*a*, 1, 2, 3) and  $1.22^{\circ}$ /cm. to the left for the second set (*b*, 1, 2, 3, 4).

In the third class of bees are to be found those which, although they exhibited fairly uniform behavior in a single set of trials, varied widely in different sets. For example, bee no. 82, in its first record (fig. 8, 82, *a*) showed a pronounced deflection which averaged  $5.81^{\circ}$ /cm. to the left. In its second set of trials (fig. 8, 82, *b*, 1, 2, 3, 4), on the contrary, it showed little tendency to turn, and the average deflection was but  $0.65^{\circ}$ /cm. to the left. An even more striking case of variation, however, was afforded by bee no. 63. In a single record of fifty-three seconds' duration (fig. 8, 63, *a*) this animal deflected, on the average,  $5.58^{\circ}$ /cm. to the left. Approximately two hours later, in a record of sixty seconds' duration (fig. 8, 63, *b*), the same animal exhibited an even greater average deflection in the opposite direction, viz.,  $7.50^{\circ}$ /cm. to the right. The range of variation presented by these two records is no less than  $13.08^{\circ}$ /cm.

In a uniform, non-directive light field, therefore, many bees exhibit a fairly constant tendency to turn toward a given side,

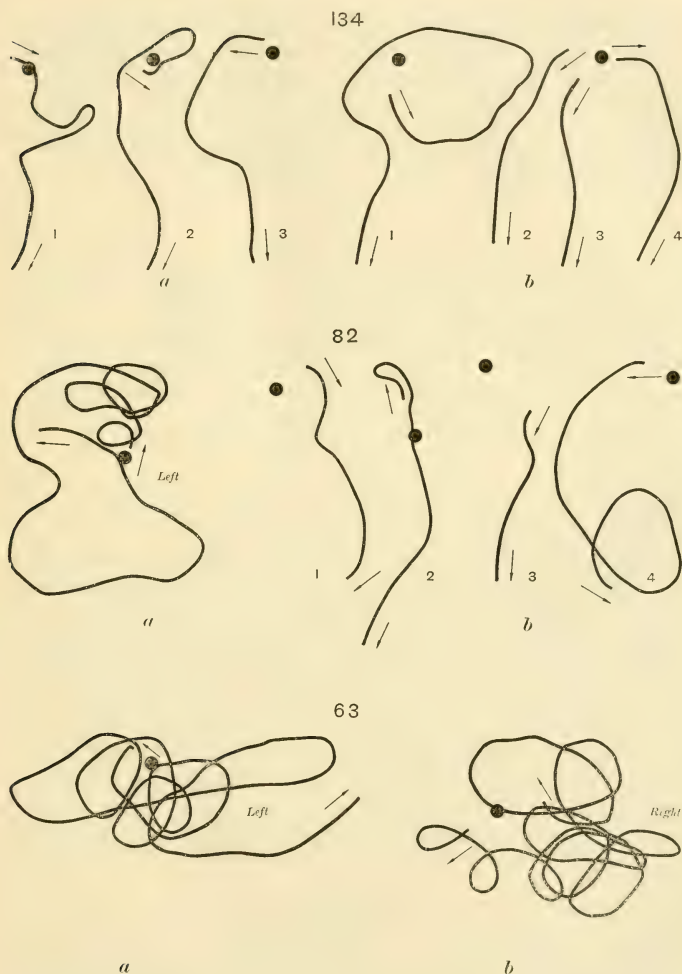


Fig. 8 Records of normal bees in non-directional light. *a*, records of the first set of trials; *b*, records of the second set of trials. Bee no. 134 exhibited little tendency to deflect in either direction. Bees nos. 82 and 63 varied widely in their average deflections in the two sets of trials.

others display little or no such tendency, while still others vary widely in their deflections from time to time. Since the animal moves in a uniform environment, the conspicuous asymmetry of response so frequently noted must be attributed to internal factors. Such factors are, for the most part, probably quite independent of light. A more detailed discussion of these will be presented in a subsequent section of this paper.

#### *4. Total darkness*

If internal factors are responsible for the asymmetric responses of bees in non-directive illumination, a similar behavior should be exhibited in the total absence of photic stimulation. Such was indeed the case. Animals creeping on smoked paper, in total darkness, showed the same conspicuous tendencies to loop and turn as did animals in non-directive light (fig. 16). The data here referred to were taken in connection with experiments conducted for a different purpose. They are, therefore, not sufficiently extensive to establish more than a similarity to the behavior exhibited in non-directive light.

Responses essentially like those of bees in total darkness have also been described by Pouchet ('72) for the larvae of *Lucilia caesar*, Davenport ('97) for the amoeba, and Frandsen ('01) for the garden slug *Limax*. Frandsen's observations in particular bear a striking resemblance to those which I have just described for the honey-bee in non-directive light. Thus he found that while most of his animals looped in a fairly constant fashion to the right or left, a few were extremely variable, while still a few others moved in rather straight courses. The responses of creeping bees in the total absence of photic stimulation are, therefore, very similar to those observed for other animals under the same conditions.

#### *5. Summary*

In the preceding pages certain responses of normal bees have been described in considerable detail, but only as a prerequisite to an adequate understanding of the behavior of the animals

when one eye is blackened. The features of behavior which are important in this connection may be summarized as follows:

1. In the honey-bee, light tends to induce activity; darkness, to inhibit it. This response is dependent upon the continuous action of photic stimulation.

2. Isolated worker bees in an active condition exhibit strong positive phototropism when flying or creeping. Temporary suppressions of this response may occur, however.

3. Normal bees when creeping in non-directive light usually exhibit pronounced asymmetrical responses of constant or variable index. Since essentially the same responses occur in total darkness they are not fundamentally dependent upon photic stimulation. They are probably, therefore, conditioned largely by internal factors.

## VI. BEHAVIOR OF BEES WITH ONE EYE BLACKENED

### 1. *Directive light*

The previous investigations of circus movements have pointed unmistakably to the generality of these responses among phototropic arthropods. Positive animals with one eye covered tend to circle toward the functional eye; negative animals, under the same conditions, tend to circle away from the functional eye. The honey-bee exhibits a striking positive phototropism. When one eye is blackened, therefore, we should expect the bee to circle toward the remaining functional eye. Such is indeed the case, as Axenfeld ('99, p. 374) has previously shown.

In my own experiments, bees thus operated upon were no longer able to creep in a straight course toward a source of illumination. Instead, their progress thither was marked by repeated loops. If the right eye was blackened, the bee looped to the left; if the left eye was blackened, it looped to the right. Moreover, it was possible by blackening one eye, then removing the black and blacking the other eye, to cause a single individual to perform circus movements first in one direction, then in the opposite direction.

The above experiment was carried out on five bees. In general, all of these animals looped more or less markedly toward the functional eye as they crept toward the light source. This tendency, moreover, was not confined to the period immediately subsequent to the operation of blackening the eye, as the experiment clearly demonstrates. The first records of these bees with their left eyes blackened were taken in the evening between 6 and 7 P.M. No further tests were made until the following day at 11:30 A.M. Yet the behavior at the end of this seventeen-hour period was practically the same as it had been before. One bee, it is true, showed considerable improvement. In the other four animals, however, the two sets of records were indistinguishable. In the absence of experience, therefore, the performance of circus movements remains a permanent feature of behavior.

Of the five bees tested, the most pronounced and uniform exhibition of circus movements was displayed by bee no. 5. Its records are almost diagrammatic in their close approximation to the theoretical expectation. Records of this animal are reproduced in figure 9.

Not all of these animals, however, yielded such striking results. Some individuals were found which manifested little or no tendency to deviate toward the functional eye, except in the area immediately beneath the lamp, where the illumination was essentially non-directive. Thus, bee no. 4, with its right eye blackened, circled toward the left in the usual manner. But a few hours later, when the black had been removed from the right eye and the left eye painted over, it exhibited little or no tendency to circle toward the right (fig. 10, *B*). The explanation at once suggests itself, that in such cases the eye was imperfectly covered, and hence not absolutely free from stimulation. This may be correct. As will be shown later, however, there are also a variety of other circumstances which might account for such behavior.

The tendency to circle toward the blackened eye was not frequently encountered in the reactions of bees to directive light. No instance of it occurred in the experiment described above, although it was occasionally met with in other experiments. A



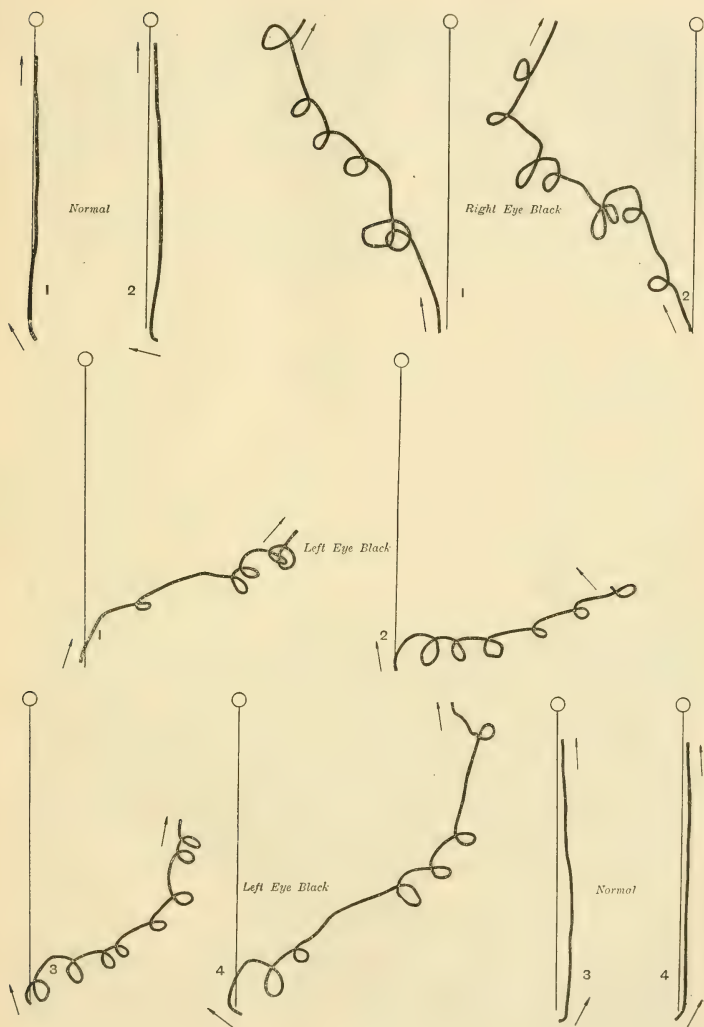


Fig. 9 Consecutive records of a bee in directive light, showing the effect of blackening first one eye, then the other.

single record of this kind is shown in figure 10, A. This was obtained from an animal which subsequently became wholly unreactive. Its aberrant tendencies may, therefore, have been due to an abnormal condition. In any case it is significant that, although the bee looped toward the covered eye, yet it progressed toward the light source. Consequently, this was not a case of reversal of phototropism.

Instances somewhat similar to the one last mentioned have been described by Dolley ('16, p. 373) for the butterfly *Vanessa*.

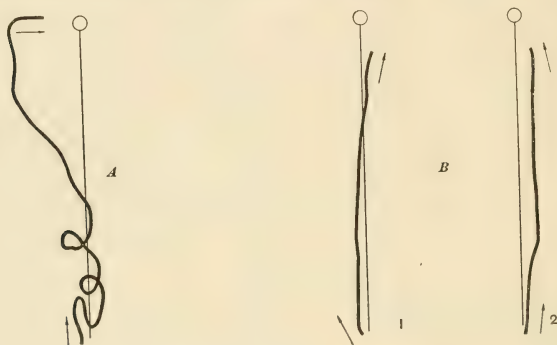


Fig. 10 A. Record of a bee in directive light, showing loops toward the blackened eye. B. Two records of a bee in directive light which showed no deflection, although the left eye was blackened.

Although positive to light, this insect with one eye blackened occasionally turned toward the covered eye instead of toward the functional eye. Possibly results of this sort are to be attributed to the effect of contact stimulus afforded by the covering of the eye, as indicated by Dolley ('16, pp. 394-399). This will be discussed more fully in a subsequent portion of the paper.

## 2. Non-directive light

*a. Amount of turning.* In non-directive light, bees with one eye blackened tended, in general, to turn more or less pronouncedly toward the functional eye. As was to be expected, the course

taken by the animals under these conditions assumed no specific direction. They either continued to circle in a fairly limited area or proceeded in a looping fashion in any direction whatever. The variability of this response, moreover, was much greater than in the case of the response to directive light. Thus, a number of animals circled almost continuously toward the covered eye in non-directive light, while still others varied, circling sometimes toward the covered eye, sometimes toward the functional eye. This was doubtless true for much the same reasons that normal bees also exhibited a greater variability of response in non-directive light.

Circus movements attendant upon the elimination of one photoreceptor undoubtedly represent the orienting process of an asymmetric animal. The specific photic stimulus, therefore, which produces these reactions must be identical with that which effects orientation in the normal animal. Whatever the nature of this stimulus be, moreover, it is afforded by both directive and non-directive light, since circus movements occur in either situation. What is the nature of this orienting stimulus? Perhaps the best method of demonstrating the dependence of a particular response upon a certain stimulus is to show that the intensity of the response varies with the intensity of the stimulus in question. It seemed possible to attack this problem, therefore, through a study of the relationship existent between the amount of turning displayed by an animal with one eye blackened and the intensity of the illumination to which it was subjected.

Non-directive illumination was chosen in preference to directive illumination because of the simpler experimental conditions which the former affords. In directive light, every movement of the entire animal is accompanied by more or less complicated changes not only in the intensity of the stimulation received, but also in the area of the eye stimulated. As an animal with one eye covered moves toward a light source, the stimulation of the functional eye steadily increases. As it loops toward this eye, however, this steadily increasing stimulus is subjected to rapid and transitory fluctuations. When the animal begins to loop, the functional eye is first turned away from the light, resulting

in a rapid decrease of photic stimulation. As the loop is completed, the photoreceptor in turn experiences an increase in stimulation. In non-directive illumination such as was employed in the present experiments, however, these complications are largely avoided. Photic stimulation here is maintained at a fairly uniform and constant intensity over the entire surface of the compound eye.

Two slightly different types of experiment were performed. The procedure in the first type was as follows. Bees were collected from flowers in the morning between 8 and 10 o'clock, brought into the laboratory and prepared for experimentation. One and two hours later, respectively, they were given single trials in the directive light area. On a basis of these records, individuals of abnormal tendencies were discarded, and those evincing the greatest accuracy of orientation were selected.

An hour or so later, the selected bees were tested in non-directive light of 957 mc. Two sets of records, about one hour apart, were made of each animal. Each set was composed of one to several records, the aggregate duration of which, in general, was between thirty and sixty seconds. An examination of the records showed clearly whether the individual was normally right-handed, left-handed, or variable in its deflection in non-directive light. These results determined which eye should be blackened. If, for example, a bee normally circled to the right, the right eye was covered. Whatever influence was exerted by photic stimulation, therefore, would tend to force the animal toward the left. In this manner, responses which might otherwise have been mistakenly attributed to photic stimulation were to some extent eliminated.

The operations of blackening the eye were carried out in the late afternoon of the first day of experimentation, in accordance with the technic previously described. On the following morning, before resuming experimentation, it was not infrequently necessary to discard a few additional animals either because of extreme weakness or occasionally death as a result of the operation.

The majority of bees usually appeared quite normal, however, and were subjected to several series of trials in non-directive

light. Throughout a single series of consecutive trials, or, as I shall call it, a determination, one intensity of light only was employed. But in the total number of determinations the more intense illumination of 957 mc. and the less intense of 24 mc. were used an equal number of times. The animal to be tested was first removed from the dark box and exposed from half a minute to several minutes in the intensity of light in which it was to be tried. This was usually sufficient to activate the animal thoroughly, and several records were then made in the non-directive light chamber. In case the bee failed to respond to photic activation, recourse was had to mechanical stimulation. The cage was tapped or even shaken fairly vigorously until locomotion was induced. This procedure seldom failed to elicit activity. When it did fail, it was usually necessary to discard the animal altogether.

The number and duration of the records comprising a single series or determination varied widely even in the same animal. If the bee quickly encountered the side wall of the light chamber, records were short, and a number had to be taken. If, on the contrary, the animal kept well toward the center of the floor of the apparatus, one or two records were quite sufficient. In cases of great variability of response or unusual departures from the general, expected behavior, additional trials were made, on the assumption that a greater number would more accurately express the average tendency of the animal. Single trials seldom exceeded thirty seconds, and were often much shorter. Occasionally, however, records of forty-five seconds, sixty seconds, or even slightly greater durations were taken. The aggregate duration of the trials comprising a single determination, for one intensity of light, was usually in the neighborhood of thirty or sixty or ninety seconds. The adoption of any more uniform period for all animals, at all times, was quite impossible.

Upon completion of a series of trials in one intensity of light, the bee was returned to the dark box. Here it was allowed to remain for a period of about fifteen minutes to one hour. In the earlier experiments the longer period was practiced; in subsequent experiments, the shorter. After this period in the dark, the ani-



mal was subjected to a second set of trials of the same aggregate duration as the first, but in the other of the two light intensities. The order in which the two intensities of illumination were employed was varied from time to time. Sometimes the first determination was made in the more intense light; the second, in the less intense. Sometimes the reverse order was observed.

A single series of records in one intensity of non-directive light together with the corresponding series in the other intensity constitute what I shall term a pair of determinations. The protocol of such a pair of determinations on bee no. 42 is given in table 3. Four or five pairs of determinations were usually made on each individual of an experiment in the course of a day, beginning

TABLE 3

DETERMINATION FOR NON-DIRECTIVE LIGHT OF 24 MC.			DETERMINATION FOR NON-DIRECTIVE LIGHT OF 957 MC.		
Number of record	Hour of record	Duration of record	Number of record	Hour of record	Duration of record
		<i>seconds</i>			<i>seconds</i>
4	1:47 p.m.	30	1	1:32 p.m.	31
5	1:47½ p.m.	30	2	1:32¾ p.m.	30
6	1:48 p.m.	30	3	1:33¼ p.m.	30
Totals .....		90			91

between 8 and 9 o'clock in the morning and concluding between 4 and 5 in the afternoon. The bees often seemed to become sluggish in the late afternoon. Whether this was due to fatigue or a natural rhythm of activity from day to night, I am unable to say. This phenomenon, however, led me to abandon any attempt to continue experimentation much after 5 o'clock.

On the third and concluding day of the experiment, the scheme of the second day was again carried out as far as possible. Bees usually survived the first two days of experimentation, and in case they did not, the data on them were discarded. A number of individuals, however, failed to survive in fit condition for the trials of the third day, and still others had to be discarded in the course of the day, although in both cases the results were counted.

Some of the more vigorous animals survived not only a third day of experimentation, but lived on for three or four days, and in a few instances even longer. Although no further trials were made with such bees, they were kept and, as far as possible, records of their subsequent longevity taken.

Having described the first type of non-directive light experiment in considerable detail, the second type may be described very briefly. It differed from the first only in the method of making pairs of determinations. In this case, the two determinations of a pair were made during the same period of time,

TABLE 4

DETERMINATION FOR NON-DIRECTIVE LIGHT OF 24 MC.			DETERMINATION FOR NON-DIRECTIVE LIGHT OF 957 MC.		
Number of record	Hour of record	Duration of record	Number of record	Hour of record	Duration of record
		<i>seconds</i>			<i>seconds</i>
2	1:43 p.m.	11	1	1:41 p.m.	30
4	1:51 p.m.	23	3	1:48 p.m.	44
5	1:53 p.m.	40			
7	1:59 p.m.	30	6	1:56 p.m.	30
Totals .....		104			104

instead of an appreciable interval apart. The bee was first tested in one intensity of light, then within a minute or so in the other intensity, then again in the first, and so on until a series of one to five records had been completed for each intensity. Care was exercised, however, even with this rapid alternation of intensities, always to expose the animal for thirty to sixty seconds in a given intensity before subjecting it to a trial in the same. The following protocol from bee no. 83 (table 4) will illustrate this method of making determinations.

In both types of experiment, there were obtained for each bee a number of pairs of determinations, usually four to ten, depending upon the longevity of the individual. The records of each

determination have been computed collectively in the manner already described. Single values have thus been derived which express the average deflection, or tendency to turn, exhibited by the animal in each determination. When the turning was chiefly toward the blackened eye, the sign of these values is negative; when chiefly toward the functional eye, it is positive. If, now, the value of each determination in 24 mc. light be subtracted from the corresponding one in 957 mc. light, differences will be obtained which should answer conclusively the question of relationship between the amount of turning and the intensity of photic stimulation.

The differences obtained in the manner just described I shall designate as *d*. A given value of *d* may be negative or positive. If it be negative, it signifies one of the two following possibilities:

1. The bee turned *more toward the blackened eye in an illumination of 957 mc. than it did in one of 24 mc.*

For example, in the second pair of determinations on bee no. 32 (table 2),

$$\begin{array}{rcl} 957 \text{ mc.} & 24 \text{ mc.} & \\ -13.28^\circ/\text{cm.} & - (-9.22^\circ/\text{cm.}) & = -4.06^\circ/\text{cm.} \end{array}$$

2. The bee turned *less toward the functional eye in an illumination of 957 mc. than it did in one of 24 mc.*

For example in the second pair of determinations on bee no. 23,

$$\begin{array}{rcl} 957 \text{ mc.} & 24 \text{ mc.} & \\ +6.44^\circ/\text{cm.} & - (+8.92^\circ/\text{cm.}) & = -2.48^\circ/\text{cm.} \end{array}$$

If, however, the value of *d* be positive, it signifies one of the two following possibilities:

1. The bee turned *less toward the blackened eye in an illumination of 957 mc. than it did in one of 24 mc.*

For example, in the first pair of determinations on bee no. 31,

$$\begin{array}{rcl} 957 \text{ mc.} & 24 \text{ mc.} & \\ -3.87^\circ/\text{cm.} & - (-7.14^\circ/\text{cm.}) & = +3.27^\circ/\text{cm.} \end{array}$$

2. The bee turned *more toward the functional eye in an illumination of 957 mc. than in one of 24 mc.*

For example, in the first pair of determinations on bee no. 21,

$$\begin{array}{rcl} 957 \text{ mc.} & 24 \text{ mc.} & \\ +29.41^\circ/\text{cm.} & - (+15.84^\circ/\text{cm.}) & = +13.57^\circ/\text{cm.} \end{array}$$

If the great majority of  $d$  values are of the first category, viz., negative, we may conclude that the animal experiences a greater impulse to turn toward the functional eye in an illumination of 24 mc. than it does in one of 957 mc. If equal numbers of negative and positive values occur, there is no relation between the intensities of photic stimulation employed and the amount of turning. If, however,  $d$  is generally positive, we may conclude that the tendency to turn toward the functional eye increases if the intensity of photic stimulation is sufficiently increased.

Experimentation soon demonstrated that the only satisfactory solution of the problem was to be had through a statistical treatment of large numbers of data. Even the more constant animals often varied widely from one pair of determinations to another without any apparent external cause. Therefore, a large number of bees were experimented upon and each individual was subjected to many tests, the averages of which were relied upon to indicate the general trend of behavior. In table 2 (appendix) are presented the results obtained from a careful measurement and computation of over two thousand records taken on fifty-two bees. On some individuals as few as sixteen records were taken; on others as many as seventy-four. This difference was due in part to varying longevity of individuals and in part to the fact that more favorable animals were frequently experimented with longer than less favorable ones. The determinations of approximately the first half of the animals were made on the plan of the first type of experiment, while the remainder were carried out according to the scheme used in the second type.

From the figures presented in columns F and G of table 2, it is evident at once that there is a marked preponderance of the positive  $d$  values over the negative. The ratio of the two is strikingly shown in the frequency polygon (fig. 11). Since the number of  $d$  values varied considerably with the individual, due

to the causes noted above, I have included in the polygon only the first four values for each bee, thus giving equal weight to every animal. Of the total two hundred and seven<sup>6</sup> values represented in the polygon, 81.16 per cent are positive, whereas only 18.84 per cent are negative, a ratio of over 4 to 1.

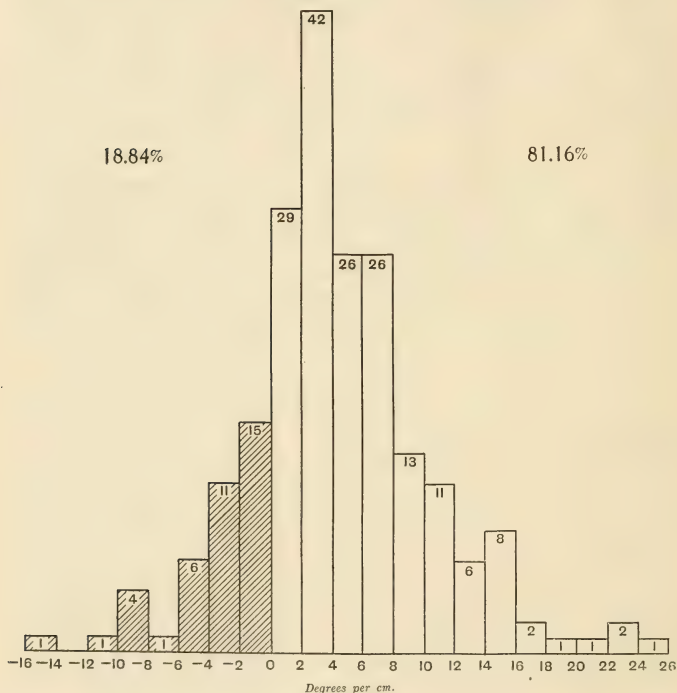


Fig. 11 Frequency polygon of the first four *d* values of fifty-two bees. The negative values are represented by the shaded areas; the positive values, by the clear areas.

<sup>6</sup> In the case of one bee it was possible to include only three values for *d*, because of a missing record. See Table 2 (Appendix), bee no. 45.



The objection might be raised that although the majority of values of  $d$  are positive, a number of them are too small to be of any significance. It is true that differences of the order of  $1^\circ/\text{cm.}$  or less might easily be attributed to slight errors in tracing the course of a bee. Errors in recording, however, are as likely to occur in one direction as the other. Such is not the case with these small values. The class of  $d$  values between  $0$  and  $-2^\circ/\text{cm.}$  contains but fifteen, while the class of  $0$  to  $+2^\circ/\text{cm.}$  numbers twenty-nine—nearly twice as many.

Moreover, the mode of the curve,  $+2^\circ/\text{cm.}$  to  $+4^\circ/\text{cm.}$ , lies well beyond these small values. Differences of this magnitude are readily detected in the records of bees which circled constantly toward the functional eye, as the pair of determinations in figure 12 demonstrate. Each record shown in the figure was of exactly thirty seconds' duration. The first two were taken three minutes apart in 24 mc. illumination, while the third and fourth were taken about twenty minutes later in 957 mc. illumination, one minute apart. The value of  $d$  in this case is  $+2.98^\circ/\text{cm.}$ —about the average modal value.

In bees exhibiting considerable variation in their deflections, however,  $d$  values, or of the modal class of even greater magnitude, are not so easily recognized without the accompanying figures. To illustrate this, I have selected a pair of determinations (fig. 13) approximating the mean value of the frequency polygon, which is  $+4.35^\circ/\text{cm.}$  As attested by the data given in connection with the figure, this animal was extremely variable in its direction of turning. All four records were taken in the brief period of eight minutes, nos. 1 and 2 being of twenty-eight seconds' duration each; nos. 3 and 4, of thirty seconds. The value of  $d$  is  $+4.09^\circ/\text{cm.}$ —a fact which does not become apparent until the records are submitted to a careful scrutiny.

The data presented in table 2 and figure 11 show clearly that bees with one eye blackened tend to turn more toward the functional eye in a non-directive illumination of 957 mc. than they do in one of 24 mc. The validity of this conclusion is confirmed by still another line of evidence. A certain number of animals were found to exhibit a very constant tendency to turn toward

the functional eye, which was always much more pronounced in the intense than in the weak illumination. In other words, these individuals always yielded positive values of  $d$  of rather high magnitude. Bees nos. 73, 83, 95, 105, and to a lesser extent numerous others afforded striking examples of such behavior. These

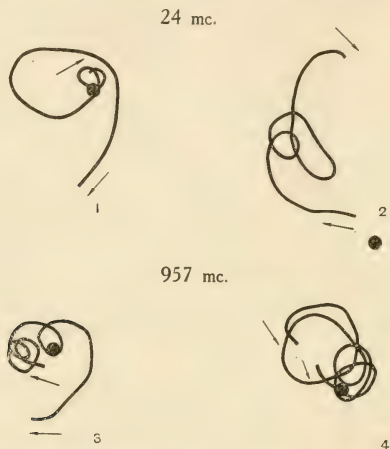


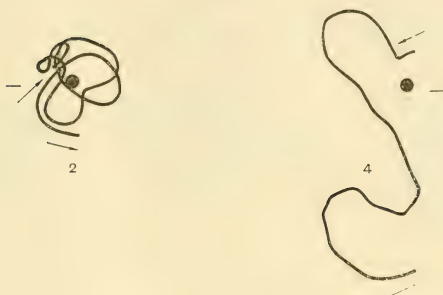
Fig. 12 A pair of determinations of bee no. 42, left eye black, non-directive light. The records are numbered in the order in which they were taken.

Number of record	24 mc. Light		Number of record	957 mc. Light	
	+Degrees turned	-Degrees turned		+Degrees turned	-Degrees turned
1	900	0	3	1440	0
2	945	0	4	1485	0
Average deflection, +9.49°/cm.			Average deflection, +12.47°/cm.		
d = +2.98°/cm.					

animals were all thoroughly vigorous individuals, surviving not only the three days of experimentation, but living on for at least two days thereafter. Two of these bees, in fact, survived no less than four days after the conclusion of the experiment.

In figures 14 and 15 are shown pairs of determinations from two of these animals. The eight records of bee no. 73 (fig. 14) were taken in the course of  $28\frac{1}{4}$  minutes, while the six records of

24 mc.



957 mc.

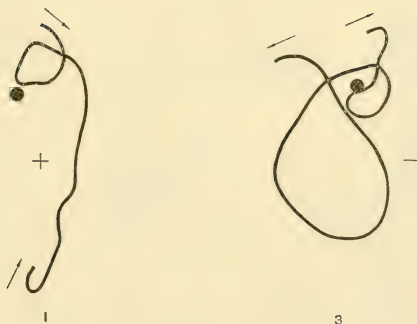


Fig. 13 A pair of determinations of bee no. 123, left eye black, non-directive light. The records are numbered in the order in which they were taken.

Number of record	24 mc. <i>Light</i>		Number of record	957 mc. <i>Light</i>	
	+Degrees turned	−Degrees turned		+Degrees turned	−Degrees turned
2	495	1170	1	630	0
4	270	450	3	225	720
Average deflection, $-3.53^{\circ}/\text{cm.}$			Average deflection, $+0.56^{\circ}/\text{cm.}$		
$d = +4.09^{\circ}/\text{cm.}$					

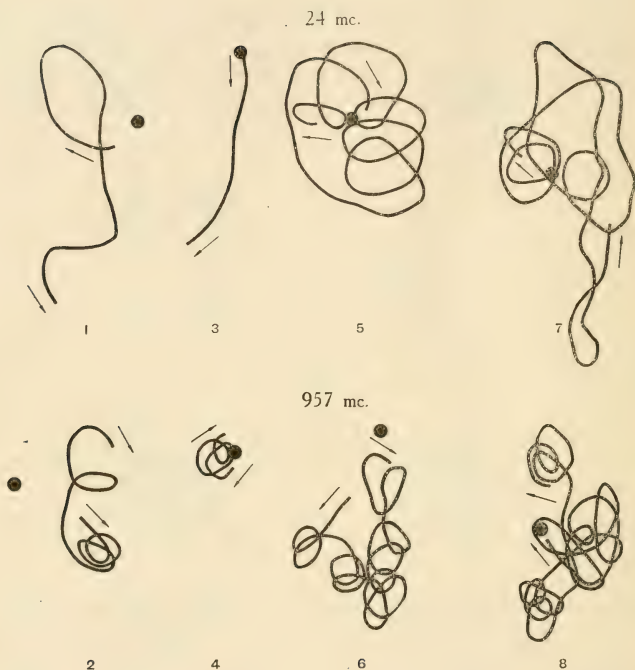


Fig. 14 A pair of determinations of bee no. 73, left eye black, non-directive light. The records are numbered in the order in which they were taken.

Number of record	24 mc. Light		Number of record	957 mc. Light	
	+Degrees turned	-Degrees turned		+Degrees turned	-Degrees turned
1	360	90	2	1485	0
3	45	0	4	990	0
5	2115	0	6	3510	0
7	2070	225	8	4005	0
Average deflection, +6.07°/cm.			Average deflection, +13.82°/cm.		
d = +7.75°/cm.					

bee no. 105 (fig. 15) required only fifteen minutes. In both figures, each record of the top row is of exactly the same duration as the corresponding one of the lower row, except records 1 and 2 of bee no. 73, which differ by one second. It would be difficult to

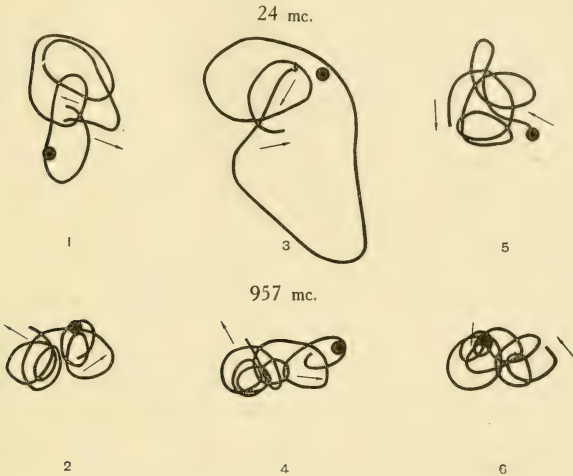


Fig. 15 A pair of determinations of bee no. 105, right eye black, non-directive light. The records are numbered in the order in which they were taken.

Number of record	24 mc. Light		Number of record	957 mc. Light	
	+Degrees turned	--Degrees turned		+Degrees turned	--Degrees turned
1	1260	45	2	1935	0
3	855	0	4	2430	0
5	1305	90	6	2520	0
Average deflection, +6.74°/cm.			Average deflection, +14.83°/cm.		
d = +8.09°/cm.					

imagine more conclusive results than those afforded by these two bees.

It might be supposed that animals would be found which would exhibit the opposite of the condition just described. Such, however, was not the case. I failed to find any individuals which continually circled more toward the functional eye in an illumi-



nation of 24 mc. than they did in one of 957 mc. Bees presenting a number of negative  $d$  values, such as nos. 22, 25, 34, 41, 43, 55, 56, 62, 66, 85, 126, and 135, with one exception, showed an equal or greater number of positive values. The exception noted was bee no. 56. Three of the four pairs of determinations obtained on this animal not only yielded negative differences, but differences of large magnitude as well. Bee no. 56, like a number of other individuals presenting a considerable number of negative  $d$  values, varied considerably in its behavior and turned chiefly toward the blackened eye. How far the disturbing factors thus evidenced account for the results is not absolutely certain, since a number of bees of apparently similar tendencies yielded positive values of  $d$ . Certainly, however, there are a number of factors, particularly in the type of experiment under consideration, which do interfere with the effect of photic stimulation. Some of these serve to intensify the response, while others tend to counteract or even completely annul it. Without attempting to minimize the significance of these negative data in the least, I believe some of them, probably all of them, find their explanation in such factors. If this be correct, the negative results obtained lie well within the range of variation which might be expected in experimental work of this sort. A more extended discussion of the factors responsible for variability of behavior in the present experiments is presented in the next section of this paper.

The evidence in general, therefore, seems to warrant the conclusion that bees with one eye blackened tend to turn more toward the functional eye in an illumination of 957 mc. than in one of 24 mc. This tendency may result in the animal's actually turning more toward the functional eye, or in its turning less toward the covered eye, depending upon the idiosyncrasies of the individual. In either case, however, with increased photic stimulation, there is an increased tendency toward the functional eye. The nature of the stimulus afforded by the apparatus employed was continuous and of almost uniform intensity, and since the circus movements of the honey-bee vary with the intensity of such stimulation, they must be dependent upon it. These con-

clusions are the exact antitheses of those reached by Dolley ('16) in his work on Vanessa. He says (p. 417): "Vanessas with one eye blackened do not move in smaller circles in strong light than they do in weak light, unless it is extremely low. On the contrary, the evidence seems to indicate that the stronger the light is the larger the circles are. These results also are not in harmony with those demanded by the 'continuous action theory.' " I shall return later to a more complete consideration of the theoretical significance of the results afforded by the honey-bee.

*b. Rate of locomotion.* Although bees with one eye blackened tend to turn more toward the functional eye in a non-directive light of 957 mc. than they do in one of 24 mc., there is no difference in the rate of locomotion in the two intensities. In table 5 are given the average velocities of thirty-four bees for each of the two light intensities employed. These figures show a considerable range of individual variation, from as low as 3.49 cm. per second to as high as 6.77 cm. per second. There is, however, no consistent difference which might be attributed to the effect of light. Eighteen of the animals showed a greater velocity in 957 mc. illumination; sixteen, in 24 mc. illumination. Unilateral photic stimulation of the intensities employed is, therefore, without effect upon the rate of locomotion.

### 3. Summary

1. Bees with one eye blackened usually loop toward the functional eye as they creep toward a source of light. Some individuals are encountered however, which display little tendency to loop, and occasionally an animal will be found which loops toward the covered eye. In the absence of experience, the tendency to loop toward the functional eye remains a permanent feature of behavior.

2. In non-directive light, bees with one eye blackened generally circle toward the functional eye, although a number are found which circle more or less toward the covered eye.

3. In a uniform non-directive illumination of 957 mc., the tendency to turn toward the functional eye is greater than it is in a similar illumination of 24 mc.

4. Since the amount of turning varies directly with the intensity of continuous photic stimulation, the turning is produced by this stimulus.

5. Unilateral photic stimulation of the intensities employed has no effect upon the rate of locomotion.

TABLE 5

A NUMBER OF BEE	B VELOCITY CM. PER SEC. 24 MC. LIGHT	C VELOCITY CM. PER SEC. 957 MC. LIGHT	D C-B
21	6.10	6.22	+0.12
22	5.10	5.12	+0.02
23	5.02	5.03	+0.01
24	4.95	5.18	+0.23
25	4.67	4.53	-0.14
31	3.77	4.23	+0.46
32	4.20	4.74	+0.54
33	5.27	5.19	-0.08
34	4.78	4.99	+0.21
36	3.49	3.63	+0.14
41	4.45	4.29	-0.16
42	4.76	4.30	-0.46
43	5.59	5.76	+0.17
44	4.94	4.90	-0.04
45	4.36	4.99	+0.63
51	4.51	4.40	-0.11
52	5.09	5.08	-0.01
53	6.77	6.53	-0.24
54	5.53	5.17	-0.36
55	6.09	5.21	-0.88
56	5.32	5.53	+0.21
62	4.82	4.59	-0.23
63	5.80	5.72	-0.08
66	5.10	5.15	+0.05
68	5.14	5.34	+0.20
72	4.69	4.87	+0.18
73	4.97	5.44	+0.47
77	4.26	3.99	-0.27
81	4.41	4.32	-0.09
82	5.25	5.29	+0.04
83	5.22	4.93	-0.29
85	4.91	4.79	-0.12
91	4.48	4.64	+0.16
92	4.49	4.51	+0.02

## VII. VARIABILITY OF PHOTIC RESPONSE

The honey-bee is remarkably constant in the strong positive phototropism which it evinces. The course of individuals creeping in directive light is a straight path toward the source. Yet, as has been shown, occasional departures from this behavior do occur. In non-directive light, moreover, the responses of normal bees are frequently extremely variable. The animal may turn markedly toward a given side in one trial, and in the next, turn quite as markedly toward the opposite side. Again, the direction of turning may be completely changed several times in the course of a single trial.

It is not surprising, therefore, that bees with one eye blackened also exhibit considerable variability of response in both directive and non-directive illumination. In non-directive light particularly, there were a number of cases in which animals turned little or not at all toward the functional eye, while there were still others in which they circled chiefly toward the covered eye. In fact, over 25 per cent of the first four pairs of determinations on the fifty-two bees, when averaged, gave negative values. These departures from the more usual tendency, to turn toward the functional eye, sometimes characterized the entire behavior of an individual; again, they appeared only spasmodically.

Thinking that some of the results above mentioned might be attributed to a loss of phototropism, either permanent or extending over a considerable interval of time, I frequently subjected the animals exhibiting them to one or more immediate trials in directive light. This, however, failed to show anything which might be construed as a loss of phototropism. The variations of response noted, therefore, must be referred to external and internal factors which are capable of modifying, in a more or less profound way, the dominating effect of unilateral photic stimulation. Such factors are of two sorts, those which are continuously effective and those which are not continuously effective, but fluctuate from time to time. Both types played so considerable a rôle in the experiments previously described, that I have felt they merited the somewhat extended analysis presented in the following pages.

### 1. *Continuous factors*

*a. Temperature and humidity.* Of the continuously operative factors, none are more important than the general conditions of temperature and humidity. These profoundly affect the activity of bees. McIndoo ('14, p. 279) says: "Climatic conditions perceptibly affect the activity of bees. When it is extremely warm, they are most active and are rarely quiet even for a few seconds. When it is moderately warm, they are less restless, and when rather cool, bees do not move freely." Again he says: "During cool weather their movements are quite sluggish, and when the humidity is high they are much less active and respond to various odors more slowly than when there is low humidity."

Precisely the same effects were noted in the present experiments. So serious did they become on several occasions that the experiment had to be abandoned. On cool, damp days bees were apt to be quite unreactive, and prolonged exposure to light often failed to induce locomotion. Considerable mechanical stimulation might call forth creeping, but it was of desultory kind and was apparently unaffected by photic stimulation. There can be no doubt, therefore, that general weather conditions considerably affect the behavior of bees toward light. Since some experiments were continued under less favorable weather conditions, it is quite probable that they account for some of the aberrancies observed.

*b. After-effects.* In making quantitative determinations in non-directive light, trials were frequently made in the two intensities in rapid succession. Although the bee was always exposed for at least thirty seconds to a given intensity before making a test, there still existed the possibility that an after effect of the first intensity might influence the behavior of the animal in the second. Thus Herms ('11, p. 215) has demonstrated an after-effect of photic stimulation in the blow-fly larva, which may manifest itself in the continued orientation of the animal for as much as fifteen to twenty seconds after the cessation of the stimulus. That such is not the case for bees, however, is very clearly demonstrated by the following experiment.



Normal bees were allowed to creep on a strip of smoked paper toward the 80-c.p. incandescent lamp. When, after creeping a distance of about 60 cm., the bee reached a point 40 cm. from the source of light, the lamp was extinguished. Not only was the current turned off, but a screen was simultaneously placed before the lamp, so that even the slight after-glow of the filaments was eliminated. After ten seconds of total darkness the light was turned on and the bee removed. Two to four records were made of each of eleven animals in this manner. In not one case did the bee fail to lose its orientation within at most a couple of seconds after the extinction of the light. Often the loss of orientation was almost, if not quite, simultaneous with the cessation of the stimulus. The deviation from the former orientation was sometimes marked by a pronounced tendency to turn toward one side, with the result that the animal crept in circles in the dark. At other times the bee merely wandered, turning first in one direction, then another. Typical records of two animals are reproduced in figure 16. There is, therefore, no after-effect of photic stimulation in the honey-bee, and this does not account for any of the irregularities observed.

*c. Failure to eliminate photoreceptor.* The difficulties in manipulating bees necessitate relying upon a single operation to eliminate completely the function of the compound eye. Although in this operation great care was exercised to cover the eye completely with a heavy coat of paint, there is a possibility that in a few cases this was not accomplished. Moreover, from the moment the animal began to recover from the anaesthetic, the covered eye was repeatedly subjected to vigorous scrapings by the front leg of the same side. While the examination of a number of animals after experimentation showed that this seldom resulted in a removal of any of the eye covering, it probably succeeded in doing so in a few cases. Occasionally, also, the varnish cracked somewhat on drying. These unavoidable failures to keep the compound eye entirely free of light, undoubtedly modified, to a greater or less extent, not a few of the results obtained.

Beside the failure to cover the eye, there is the possibility that the three ocelli of the honey-bee are concerned in its photic

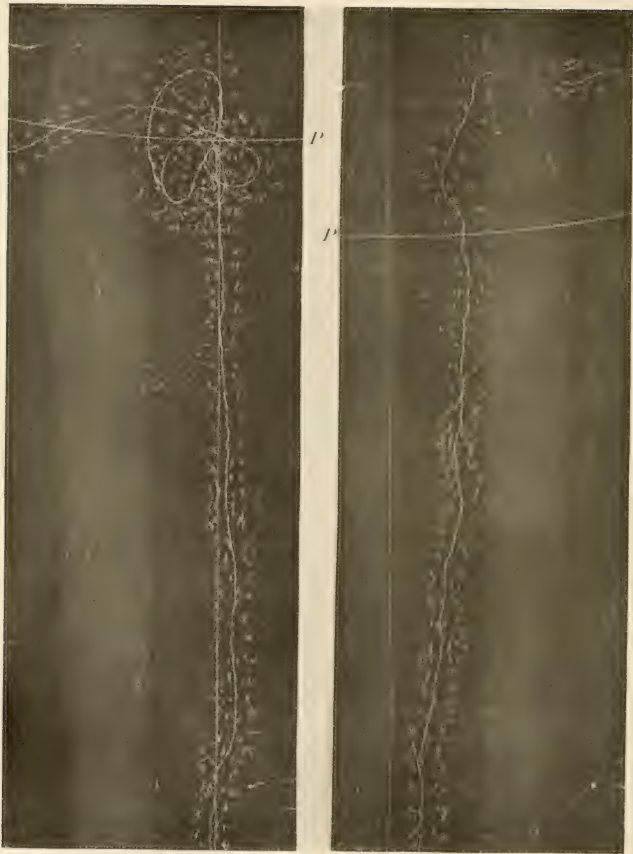


Fig. 16 Smoked-paper records of two normal bees creeping toward a light source (upward in the figure). As the animals reached the line indicated by the letter *P* the light was extinguished. Note the extremely rapid loss of orientation.

behavior. In blackening the compound eye I made no attempt to cover the ocelli, but afterward in examining the specimens used, I found that sometimes all, sometimes only one or two, at other times none of these organs had been covered. If the ocelli do exercise any considerable function, therefore, there was involved here a variable of no small magnitude.

It is also possible that in bees other portions of the body, or even the entire integument, may be photosensitive. Photodermatic sensitivity is not unknown among arthropods. It has been reported by Graber ('84) for the cockroach, by Plateau ('87) for two species of blind myriapods, and by Stockard ('08) for the walking-stick.

In order to find out if these several possibilities were affecting results, I conducted several experiments with bees both eyes of which had been carefully covered with a thick coat of 'Jap-a-lac.' On the morning after the operation, each bee was placed in a separate cage in non-directive illumination of 957 mc. Although with one or two exceptions the bees were quiet when first exposed to the light, within fifteen minutes all had become thoroughly active, showing clearly that they were not free from the activating effect of the light.

At the conclusion of the above test the same bees were individually subjected to trials in the directive light area. Here they looped and turned in a variety of ways, some circling more or less constantly toward a given side, not unlike bees with only one eye blackened. Despite most devious courses, however, they sooner or later managed to work their way to the general region of the source of light. It is quite clear, therefore, that photoreception had not been entirely abolished, although both compound eyes had been covered as carefully as possible.

Which of the several explanations advanced accounts for these results, is not certain. I am disposed to believe that the failure to eliminate the compound eyes completely was chiefly, perhaps solely, responsible. However that may be, it is certain that in this, as well as in other experiments, the incomplete suppression of photic stimulation was the source of a large amount of variation in the results obtained.

*d. Effect of contact stimulus.* The effect of the contact stimulus afforded by the blackening material on the eye and the adjacent parts of the head must also be recognized as a considerable factor in modification of photic behavior. The influence of this stimulus has been clearly demonstrated by Dolley ('16, pp. 394-397) on *Vanessa antiopa*. With one eye blackened, this butterfly, when creeping in total darkness, turned, with few exceptions, continuously toward the blinded eye. The tendency to circle was often quite pronounced, and showed little or no modification from day to day. The effect of contact stimulus on the covered eye was, therefore, antagonistic to that produced by light on the opposite, functional eye.

I tried experiments with the honey-bee similar to those carried out by Dolley on *Vanessa*. Bees with one eye blackened were allowed to creep on smoked paper in total darkness. Unfortunately, the trials were of such duration that the bee recrossed its course many times. This made it quite impossible to decipher the records, and I have not since had an opportunity to repeat the experiments. It is not unlikely, however, that the effect of contact stimulus on the eye of the bee is similar to that demonstrated for *Vanessa*. If such be the case, it probably accounts for much of the circling toward the covered eye, which was evinced by numerous individuals particularly in non-directive light.

*e. Asymmetry of the animal.* As was previously pointed out, normal bees frequently exhibited a marked tendency to turn more or less constantly toward the right or left when creeping in non-directive light. Such tendencies are doubtless due to a lack of perfect symmetry on the part of the animal. The asymmetry may be physiological; it may be anatomical. It may consist of a differential sensitivity of the photoreceptors on the two sides of the body, as Patten ('14, p. 259) has suggested, or it may be due to an inequality of any two symmetrically located elements of the neuromuscular mechanism. Under the influence of directive light, these idiosyncracies are, as a rule, continually corrected. In non-directive illumination or total darkness, however, they at once assert themselves. Since the eye to be blackened was always chosen so that the effect of photic stimulation would be opposite

to that exerted by natural asymmetry, many failures to turn toward the functional eye are probably thus accounted for.

*f. Modifiability through experience.* The work of Axenfeld, Holmes, Brundin, and Dolley has shown that a number of arthropods are able to modify their photic behavior through experience. The same is true of the honey-bee, at least in directive light, as the following experiment shows. Each of a number of normal bees, selected on the basis of the accuracy with which they oriented to directive light, had one eye blackened. On the following day those animals which exhibited a more or less pronounced tendency to loop toward the functional eye were subjected to trials (twenty to twenty-five in number) in the directive light area.

Bees which displayed little or no tendency to loop were given several trials to ascertain if their behavior was constant, and then discarded. These animals may have been able to modify their behavior almost immediately, or their failure to exhibit circus movements may have been due to an imperfect covering of the eye, or to the effect of contact stimulus.

Of those bees which did perform circus movements, records were taken about every ten to twenty minutes from 9 A.M. to 5 P.M., with the exception of about an hour at noon. Ten bees were thus tested. Four of these animals displayed a steady and marked improvement in the course of the trials. Two others showed some improvement, although considerably less than the first four. Two more of the ten improved for a time, only to regress again, so that while a number of trials near the middle of the series were somewhat modified, those at either end were much alike in the number of loops performed. The last two bees showed practically no improvement, although in one of the animals the tendency to circle was at no time very pronounced. It is quite certain, therefore, that at least some bees are able to modify their responses to directive light through experience.

The records shown in figure 17 afford a striking example of this modifiability. Although in its first trials the animal looped repeatedly as it crept toward the light, it was subsequently able to reach the light by a nearly straight course. This animal, how-



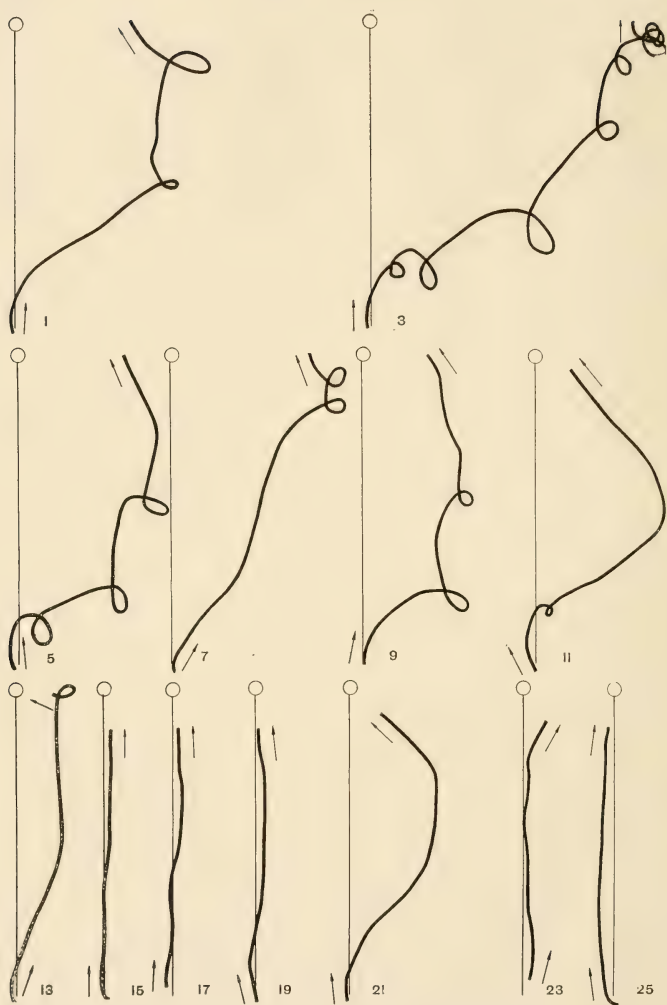


Fig. 17 Records of a bee with the left eye blackened, in directive light, showing modifiability of behavior through experience. Alternate records from a series of twenty-six are shown in the figure.

ever, as well as the others, usually circled again toward the functional eye upon reaching the non-directive region near and directly beneath the lamp. The records shown in the figure do not include this region.

Dolley ('16, p. 402) states that he observed some modification from day to day in the behavior of Vanessa in non-directive light. The following evidence seems to indicate that the same is true, to at least some extent, for the honey-bee also. In directive light, individuals with one eye covered were sometimes observed to begin to swerve toward the functional eye, only to check themselves by a sharp turn in the opposite direction. Correcting movements of this sort sometimes occurred repeatedly in a single trial, with the result that the animal reached the source of light by a much more direct course than it would have otherwise been able to follow. Precisely the same sharp turns away from the functional eye were occasionally seen in non-directive light also. It seems probable, therefore, that modifiability through experience affects the behavior of bees in non-directive as well as in directive light.

## *2. Fluctuating factors*

The variables which have thus far been discussed are those which continuously or progressively affect the behavior of bees throughout an experiment. They probably account in a large measure for such phenomena as the persistent turning toward the covered eye in certain individuals or the apparent lack of any tendency to turn at all in still others. They do not, however, explain the many sudden changes of behavior which were observed. Such variations are dependent upon factors of behavior which fluctuate from time to time. A few of these factors result from environmental changes. The majority, however, arise from changes within the organism itself.

*a. External.* In quantitative experiments every possible precaution was exercised to keep all external factors uniform, except the intensity of the light which was changed from trial to trial. This, of course, was possible to a limited extent only. The manipulation of the bees introduced varying mechanical stim-

uli which were quite unavoidable. An animal was accidentally pressed slightly in changing it from one cage to another, or, failing to react to photic stimulation, the cage had to be shaken. Again, in transferring a bee from the light to which it was subjected for activation to the center of the non-directive light chamber, it was subjected to increases and decreases of light intensity. All of these details and endless others, collectively and individually, were undoubtedly responsible for many of the sudden variations of behavior which occurred.

*b. Internal.* The chief causes of irregular variations, however, are internal. When under constant external conditions, a bee varies the direction of its turning several times in the course of a single trial, the behavior must be attributed to changes within the animal itself, the physiological states of Jennings ('06). Generally speaking, the analysis of these changing physiological states is difficult or impossible. In several instances, however, I was able to make a fairly certain diagnosis. I may cite several examples.

Bee no. 147 was subjected to the usual quantitative experiment in non-directive light. From the beginning this animal exhibited a rapid, uneasy locomotion. Its entire behavior may best be described by the word 'excitable.' In the course of the first day after the eye was blackened, five pairs of determinations were made. In 24 mc. light, the animal circled chiefly toward the covered eye, while in 957 mc., its behavior varied from one set of records to another.

On the following morning, the first pair of determinations was begun at 9:49. The bee circled markedly toward the covered eye in both intensities of light. At 12:14, a second pair of determinations was begun. In making the first trial, the bee rushed about its cage for some minutes before finally creeping up into the non-directive light chamber. When it did appear, it seemed greatly excited and crept very rapidly. As the series of records progressed, the animal circled more and more pronouncedly toward the functional eye, the locomotion grew more rapid, and a continuous buzzing began. The performance of small circles toward the functional eye in 24 mc. light was surprising, for in

all previous record sets for this intensity, the animal had shown a more or less pronounced tendency to circle toward the covered eye. In the course of the fourth trial in 957 mc. light, the bee had reached a state of intense excitation. Its turning became so rapid, and the consequent circles so small, that at length the animal tumbled over on its back. Defaecation occurred. Meanwhile it continued buzzing loudly, and, though lying on its back, managed to whirl round and round toward the functional eye.

After the trial just described, the animal was allowed to rest for two hours. At 2:44 and 4:10, respectively, two more pairs of determinations were made. The bee continued to circle markedly toward the functional eye in 957 mc. light, and more or less toward the functional eye in 24 mc. light. The 'excitement' which had characterized the previous trials, however, was absent, and the animal manifested signs of weakness and exhaustion.

In the behavior of this animal there was a sudden—even violent—increase of phototropism. This was probably due to an unusual intensification of activity. I have repeatedly observed the close correlation between these two features of behavior in bees. As a rule, the greater the activity, the more pronounced is the manifestation of phototropism. The increase of activity in this animal was produced by a state of metabolism, entailed by a collection of faeces in the intestine. That such a condition may affect the activity of bees to a marked degree is evidenced by the following statement made by Phillips and Demuth ('14, p. 12) in connection with a study of certain hive conditions in winter. "It therefore appears that the accumulation of feces (in the intestine) acts as an irritant, causing the bees to become more active and consequently to maintain a higher temperature."

In several other animals defaecation occurred in the course of a trial without being preceded by any conspicuous change of behavior. Neither the amount voided nor the force of expulsion, however, gave any evidence of long accumulation or intestinal irritation in these cases. It seems reasonably certain, therefore, that the sudden increase of phototropism exhibited by bee no. 147 was due to the accumulation of faeces in the intestine.

There were likewise certain other physiological conditions which seemed to intensify photic reactions. Thus bees, upon first recovering from anaesthesia, were frequently observed to creep in very small circles toward the functional eye. Bees, which throughout an experiment appeared physically weak, were also apt to be more intense in their positive deflections. Examples of this latter behavior were afforded by bees nos. 77 and 92. Again, individuals which appeared vigorous at the beginning of an experiment, but became weak and moribund toward the end, generally showed a progressive increase in their circus movements. For example, bee no. 91 circled rather strongly toward the covered eye at first. In the course of the experiment, the animal became weak. Correspondingly, its average deflections became more and more positive until, just before being discarded, it was turning at the rate of  $+23.82^\circ/\text{cm.}$  in 957 mc. light.

These instances demonstrate the profound manner in which internal factors are capable of modifying photic behavior. As a rule, only the change in behavior is noted. The recognition of the internal state which conditions this outward expression is possible only in extreme cases. Nevertheless, I believe that these internal factors were directly responsible for most of the sudden variations which characterized the behavior of so many bees.

Thus far we have tacitly assumed that the honey-bee is a purely reflex organism. It is not the purpose of the present paper to discuss the mooted question of psychic powers in this animal. It may be said, however, that the opinion advanced by Bethe ('98), that the behavior of bees affords no evidence of psychical attributes, has not met with extensive approbation. Forel ('07) and v. Buttel-Reepen ('07), in particular, have presented considerable evidence to show that bees are something more than mere reflex machines.

v. Buttel-Reepen ('07, p. 23) has shown that after bees have been deadened with chloroform, ether, saltpeter, puffball, etc., their memory for location entirely disappears. Subsequently they may again 'learn' the position of the hive, etc., but for the time at least, "they have forgotten everything previously known." My own observations have shown that during recovery from an



anaesthetic and in weakened or moribund conditions the photic responses of bees become more intense. Photic behavior, however, is probably largely reflex in character. It would appear, therefore, that the same conditions which occasion a loss of 'memory' or other central function and the like cause the reflex phases of behavior to appear more boldly. In other words bees, though fundamentally reflex, may possess certain rudiments of higher behavior. Under the influence of narcotics and anaesthetics or in moribund conditions, these factors cease to affect behavior, and the animal is reduced to a simple reflex condition. If this be correct, we have here an important variable to account for modifications of photic behavior.

### *3. Summary*

The variability of response displayed by bees with one eye blackened when creeping in non-directive light is never due to a permanent loss of phototropism or to after-effects of one intensity upon trials in a second intensity. It is attributable to the following causes:

- a.* Conditions of temperature and humidity.
- b.* Failure to eliminate completely the photoreceptors on one side of the body.
- c.* Effect of contact stimulus afforded by the eye covering.
- d.* Natural asymmetry of individuals.
- e.* Modifiability through experience.
- f.* Mechanical stimuli attendant upon manipulation.
- g.* Internal factors which affect behavior variously from time to time.

## VIII. NATURE OF PHOTIC ORIENTATION

### *1. Theories*

In recent years the photic behavior of lower animals has been the subject of two theories, respectively known as the 'continuous action theory' and the 'change of intensity theory.' The 'continuous action theory,' as its name implies, postulates a continuous action of light upon the organism, orientation resulting when

such action is equal on the symmetrical photoreceptors of opposite sides of the body. In its present form this theory is perhaps best summed up by Loeb ('16, p. 259). "If a positively heliotropic animal is struck by light from one side, the effect on tension or energy production of muscles connected with this eye will be such that an automatic turning of the head and the whole animal towards the source of light takes place; as soon as both eyes are illuminated equally the photochemical reaction velocity will be the same in both eyes, the symmetrical muscles of the body will work equally, and the animal will continue to move in this direction. In the case of the negatively heliotropic animal the picture is the same except that if only one eye is illuminated the muscles connected with this eye will work less energetically."

The 'change of intensity theory,' however, accounts for orientation in an entirely different manner. According to it, the process depends not upon the continuous action of light, but upon the intermittent action of rapid changes in its intensity (Jennings '04, '06, '09; Mast, '11). In positive organisms the effective stimulus is assumed to be a sudden decrease of intensity on the photoreceptor; in negative organisms, a sudden increase. A photopositive animal, such as the honey-bee, for example, orients and maintains its orientation through sudden swervings away from the side experiencing a decrease of illumination, and orientation is attained when neither eye is undergoing such a decrease. A similar explanation is applied to photonegative organisms except that the effective stimulus for them is assumed to be an increase of intensity.

There is thus a wide diversity in the explanations of orientation offered by these two theories. In the concluding pages of this paper, therefore, I propose to discuss the evidence afforded by my own experiments, as well as that afforded by the observations on circus movements in general, with a view to ascertaining which of the two theories more correctly applies to the orientation of arthropods.

*2. Orientation in the honey-bee*

As has been previously stated, the process involved in circus movements must be regarded as identical in every respect with that involved in normal orientation. The circus movement is the orienting process. A normal creeping bee may be caused to perform circus movements without having one eye blackened, if the light is merely moved so as to keep it constantly to one side of the animal. Whether the eye be blackened or the light be moved, the case is the same. The orienting process is merely prolonged, and the final attainment of orientation prevented.

The experimental data detailed in the present paper show conclusively that when one eye of a bee is blackened, the resulting circus movements are produced by the continuous action of the light upon the functional photoreceptor. In the experiments in non-directive light, the only photic stimulation afforded was one of constant, almost uniform intensity over the entire surface of the eye. Under such conditions, the animals not only performed circus movements toward the functional eye, but the amount of turning increased with an increase in the stimulus. It is clear, therefore, that the process of normal orientation, which is identical with that involved in the circus movement, must also be dependent upon the continuous action of light.

The impulses arising from this stimulation are, at least in part, transmitted to the musculature of the opposite side of the body, since upon hemisecting the brain, the bee suffers a complete loss of phototropism (Holmes, '01, p. 227). Although his experiments were not conclusive on the point, Holmes believed that the result obtained was not entirely due to "the effect of the shock of the operation, or of incidental injury to other paths of photic impulses." It must, therefore, have been due to the severance of crossed tracts or commissures which served in the transmission of such impulses. There are present in the dorsocerebrum of the bee at least three commissures in more or less intimate connection with the optic tracts (Kenyon, '96), and it is possible that these are the elements concerned. There is thus neurological as well as physiological evidence for the crossed transmission of photic impulses.

The resultant effect of these impulses on the opposite side of the body is most probably an increase in the tonus of the extensor muscles. I have no direct evidence on this point in the case of the honey-bee. However, Holmes ('05) and Holmes and McGraw ('13), in experiments dealing with unilateral stimulation of photopositive insects, frequently observed that the legs on the side away from the stimulated eye were strongly extended, while those on the same side exhibited a pronounced flexion. Recently, Garrey ('17) has published an account of numerous experiments in which the same phenomenon was observed. In such animals it is clear that orientation is effected through a difference in the posture and not through a difference in the speed of the legs on the two sides of the body. This conclusion is further substantiated by the results obtained by Dolley ('17) on Vanessa. Careful measurement of the velocity of these butterflies showed that they did not creep faster in a very intense illumination than they did in a fairly weak one. From the above observations, therefore, it is clear that in many insects orientation is effected through changes of tension in the leg muscles. As previously stated, I have not been able to observe any constant and pronounced difference in the muscular tension on the two sides of the body in the honey-bee, although I have made only casual observations in this direction. I do believe that orientation is produced in this manner, however, and that the failure to detect it was due to the slight degree of the tensions together with the extreme rapidity of their execution.

This attempt to analyze the process of orientation in the honey-bee is, of course, far from complete. Certain features of it, however, may be defined with reasonable certainty. Thus it is clear that the stimulus regulating photic orientation is continuous and not intermittent. Furthermore, it appears to be essential that at least some of the impulses arising in the eye be transmitted to the opposite side of the body, where they probably regulate the tonus of the extensor muscles. As far as it is known, therefore, the process of orientation in the honey-bee is in strict conformity with the 'continuous action theory.'

### 3. *General evidence*

In experimenting with locomotor organisms, it is not an easy matter to regulate absolutely the conditions of photic stimulation. Thus, a photopositive animal as it moves toward the light is acted upon continuously by the light. But it is also subjected not only to a gradual increase of intensity with every forward movement, but also to sudden decreases and increases with every lateral deviation, however slight. Whether the orientation of the organism, therefore, is effected through a continuous action of the stimulus or only through sudden changes in its intensity from time to time, is frequently difficult to determine with certainty. This difficulty in separating the two conditions experimentally has led to much confusion in solving the problem of orientation. Among arthropods, however, there is a growing body of evidence tending to show that orientation is produced by continuous photic stimulation and not by intermittent changes of intensity.

No stronger evidence is to be found in this connection than that afforded by the general phenomenon of circus movements. The importance of this evidence has been repeatedly emphasized by Parker ('03, '07), Loeb ('06, '13), Bohn ('09 a, b), Holmes and McGraw ('13), Garrey ('17), and others. Parker ('07, p. 548), in reviewing one of the earlier expositions of the 'change of intensity theory' (Jennings, '06), states the case clearly when he says, "If the modern tropism theory were as weak as Jennings would have us believe, the experimental evidence upon which it rests ought easily to be explained away. Yet it has always seemed to the reviewer that the characteristic circus movements performed by animals immersed in a homogeneous stimulant, but with sense organs unilaterally obstructed, are explainable only on the basis of this theory."

This statement is certainly justified by the facts. Circus movements seem quite incapable of explanation in terms of the 'change of intensity theory' of orientation. Let us examine the case of a photopositive arthropod, with the right eye blackened, as it creeps toward a source of light. From experiment we know



that such an individual usually loops to the left. As it does so, however, the functional eye experiences a pronounced decrease of stimulation during the first half of each loop. According to the 'change of intensity theory,' such decreases should result in swerves toward the opposite side. Such, however, do not occur, as a rule. The animal, instead, completes the loop. It, therefore, does not respond very strongly to a decrease of intensity. If it did, the performance of circus movements would be quite impossible. If we assume, however, that the looping is produced by the continuous inequality in the stimulation received by the two eyes, the asymmetry of response becomes intelligible at once. For, in an animal with one eye blackened, the functional eye, even when turned farthest from the light, is the recipient of some stimulation, whereas the covered eye receives none whatever.

It may be objected, however, that many positive arthropods with one eye blackened are able to overcome their tendency to circle in directive light, and that this is a response to the decrease of intensity on the functional eye at the beginning of each loop. Such may well be the case, as Holmes ('05, p. 345) has suggested. This is a matter for further experiment to decide. In any case, however, circus movements must be regarded as an established phenomenon of general occurrence among this group of animals. The significant thing, therefore, to an understanding of orientation is to discover what is effective in producing these reactions rather than what is effective in modifying them.

The evidence afforded by circus movements in directive light as to the nature of the stimulus concerned in their production is thoroughly corroborated by the results obtained in non-directive light. Under the conditions of non-directive illumination employed by Holmes and McGraw ('13) and by myself, an animal is absolutely free from any pronounced or consistent changes in the intensity of the stimulus to which it is subjected. Moreover, the experiments of Dubois ('86) on the beetle *Pyrophorus* furnish a case in which there is no possibility whatever of intensity changes playing a significant rôle in orientation, since the source of light is within the animal itself. Yet in all these cases the elimination of one eye was usually followed by typical circus

movements toward the functional eye. In the absence of significant intensity changes, these responses must have been produced through the continuous action of light. The correctness of this conclusion is further attested by the fact that bees with one eye blackened tend to circle more toward the functional eye in a non-directive light of high intensity than in one of low intensity. The response may thus be made to vary with the intensity of a constantly acting stimulus.

Bohn ('09, a, '09 b) has suggested circus movements as a criterion for tropisms. Certainly, if the photic orientation of an animal is the result of a continuous action of the light on both eyes, as the tropism hypothesis postulates, the elimination of one eye should produce circus movements. The form of response will, of course, be subject to the peculiarities of locomotion. However, the elimination of the photoreceptors on one side of the body should result in a more or less asymmetrical response toward or away from that side, depending upon the index of phototropism. A failure to obtain such responses means either that the orienting stimulus does not consist in the continuous action of light or that modifying factors are present which interfere with the expected response. As stated in the introductory pages of this paper, the failures to obtain circus movements which have thus far been reported, are, I believe, to be attributed to the latter rather than to the former cause.

In conclusion, we may say that circus movements, both in directive and non-directive illumination, are produced by the continuous action of light and not by intermittent changes in its intensity. This, together with the general occurrence of circus movements among arthropods and the close relationship of such responses to normal orientation afford strong evidence that in this group of animals photic orientation is normally produced through the continuous action of light. This does not mean that photosensitive arthropods do not respond to sudden changes in illumination. They undoubtedly do. The orientation of the body toward or away from a source of light, however, cannot be fundamentally the result of such responses.

## IX. GENERAL SUMMARY AND CONCLUSIONS

1. Light exerts a kinetic influence upon the honey-bee; that is, it tends to induce activity. In its absence, on the other hand, activity is either greatly reduced or entirely lacking.

2. Isolated worker bees, in an active condition, exhibit strong positive phototropism when flying or creeping. Temporary suppressions of this response may occur, however.

3. Normal bees when creeping in non-directive light usually exhibit pronounced asymmetrical responses of constant or variable index. Since essentially the same responses occur in total darkness, they are not fundamentally dependent upon photic stimulation. They are probably, therefore, conditioned largely by internal factors.

4. Bees with one eye blackened usually loop toward the functional eye as they creep toward a source of light. Some individuals are found, however, which display little tendency to loop, and occasionally an animal loops toward the covered eye. In the absence of experience, the tendency to loop toward the functional eye remains a permanent feature of behavior.

5. In non-directive light, bees with one eye blackened generally circle toward the functional eye, although a number are found which circle more or less toward the covered eye.

6. Although subject to considerable variation, bees with one eye blackened tend, in general, to circle more toward the functional eye in non-directive light of 957 mc. than in one of 24 mc. This tendency may be manifested in:

*a.* A lesser amount of turning toward the covered eye in the intense light than in the less intense one.

*b.* A greater amount of turning toward the functional eye in the more intense light than in the less intense one.

7. Since circus movements not only occur in a uniform, non-directive light field, but also vary in amount with the intensity of the light, they are produced by continuous unilateral stimulation.

8. In bees with one eye blackened, the rate of locomotion, unlike the amount of turning, is not dependent upon the intensity of photic stimulation.

9. The variability of response displayed by bees with one eye blackened, when creeping in non-directive light, is never due to a loss of phototropism or to after-effects of one intensity upon trials in a second intensity. It is attributable to the following causes:

- a.* Conditions of temperature and humidity.
- b.* Failure to eliminate completely the photoreceptors on one side of the body.
- c.* Effect of contact stimulus afforded by the covering of eye.
- d.* Natural asymmetry of individuals.
- e.* Modifiability through experience.
- f.* Mechanical stimuli attendant upon manipulation.
- g.* Internal factors which may affect behavior from time to time, but not necessarily continuously.

10. Photic orientation in the normal honey-bee is effected through the continuous action of light on both photoreceptors.

11. The following considerations afford strong evidence that among arthropods generally, orientation to light is effected through the continuous action of the stimulus rather than intermittent changes of its intensity.

*a.* Circus movements are of general occurrence among phototropic arthropods.

*b.* The process involved in circus movements is identical with that involved in normal orientation.

*c.* Circus movements in directive light are explainable only on the assumption of continuous photic stimulation.

*d.* Circus movements are performed under conditions of non-directive illumination where the only stimulus afforded is one of approximately constant intensity.

12. Circus movements, as Bohn has suggested, furnish a criterion for testing the 'continuous action theory' of orientation. The failure of the test, however, does not necessarily invalidate the theory.

*Postscript.* The preparation of this paper has been much retarded by the absence of the author, who is still in government service in France. Hence it has not been possible to include in the discussion Garrey's recent paper (*Jour. Gen. Physiol.*, vol. 1,

p. 118), in which he has shown that the diameter of the circle in circus movements varies with changes in light intensity, nor Loeb's most recent volume on "Forced Movements, Tropisms, and Animal Conduct."

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XI. APPENDIX (TABLE 2)

A	B	C	D	E	F	G	H	I
NUMBER OF BEE	NORMAL BEE AV. ° PER CM. TO RIGHT 957 MC. LIGHT	NORMAL BEE AV. ° PER CM. TO LEFT 957 MC. LIGHT	ONE EYE BLACK AV. ° PER CM. TURNED 24 MC. LIGHT	ONE EYE BLACK AV. ° PER CM. TURNED 957 MC. LIGHT	+ VALUES OF E - D OR d	- VALUES OF E - D OR d	DURATION OF REC- ORDS IN SECONDS 24 MC. LIGHT	DURATION OF REC- ORDS IN SECONDS 957 MC. LIGHT
21		8.02	+15.84	+29.41	13.57	7.38	30	30
		10.63	- 3.00	+19.33	22.33		30	30
			- 3.22	+ 2.91	6.13		60	60
			- 2.35	+ 3.34	5.69		52	60
			+ 6.96	+13.59	6.63		60	60
			+ 5.06	- 2.32			58	60
			- 0.59	+ 2.70	3.29		90	80
			- 3.62	+14.72	18.34		49	47
			- 1.58	+ 0.53	2.11		70	80
22	0.19 3.00		- 7.83	- 0.74	7.09	4.04	30	19
			+ 0.96	- 3.08			20	22
			- 5.51	+ 0.40	5.91		30	21
			- 4.41	0.00	4.41		16	16
			- 3.65	- 6.37			60	60
			- 0.72	- 1.54			0.82	42
			- 5.92	- 7.32			1.40	76
			- 0.32	+ 1.45	1.77		27	33
23	10.88	2.22	+ 9.72	+13.50	3.78	2.48 2.72	30	30
			+ 8.92	+ 6.44			60	60
			+10.59	+ 7.87			30	22
			+ 5.10	+22.65	17.55		63	60
			+ 0.32	+11.24	10.92		60	48
			- 1.18	+ 9.49	10.67		38	29
			+ 5.71	+16.33	10.62		60	60
			+ 9.96	+25.12	15.16		68	47
24		12.45	+ 7.19	+17.86	10.67	8.17	30	30
		25.58	+12.80	+27.01	14.21		30	30
			+ 2.17	+12.11	9.94		55	60
			+ 1.77	+17.73	15.96		30	30
			- 2.03	+ 4.36	6.39		60	60
			- 7.02	0.00	7.02		60	60
			-11.80	- 6.37	5.43		60	60
			- 7.08	- 5.66	1.42		60	60
25		8.34	+13.49	+ 5.32		8.17	30	30
		10.09	- 5.51	+ 6.56	12.07		30	21

XI. APPENDIX (TABLE 2)—*Continued*

A	B	C	D	E	F	G	H	I
NUMBER OF BEE	NORMAL BEE AV. ° PER CM. TO RIGHT 957 MC. LIGHT	NORMAL BEE AV. ° PER CM. TO LEFT 957 MC. LIGHT	ONE EYE BLACK AV. ° PER CM. TURNED 24 MC. LIGHT	ONE EYE BLACK AV. ° PER CM. TURNED 957 MC. LIGHT	+ VALUES OF E - D OR D	- VALUES OF E - D OR D	DURATION OF REC- ORDS IN SECONDS 24 MC. LIGHT	DURATION OF REC- ORDS IN SECONDS 957 MC. LIGHT
25			+ 1.63 - 9.75 - 2.81 - 7.27 - 6.90 - 3.68	- 7.24 - 3.76 - 6.57 - 3.25 - 6.63 No reac- tion	5.99   4.02 0.27	8.87  3.76	22 60 60 30 60 36	28 55 70 30 70
31	5.05 5.04		- 7.14 0.00 + 3.53 - 3.35	- 3.87 + 3.02 + 1.05 + 1.73	3.27 3.02  5.08	2.48	63 50 71 37	48 45 74 45
32	9.34 12.55		-13.71 - 9.22 -12.17 -14.12	-12.92 -13.28 - 8.86 - 8.85	0.79  3.31 5.27	4.06	60 30 60 60	60 30 60 60
33	5.31	8.59	+ 1.92 + 5.08 - 2.12 - 6.89	- 1.80 + 7.25 + 5.08 + 1.50	2.17 7.20 8.39	3.72	78 60 58 33	70 64 58 29
34	2.34 7.66		-19.36 -24.85 -18.49 -22.05	-21.92 -16.82 -16.84 -23.68	8.03 1.65	2.56  1.63	60 50 60 60	60 60 60 60
36	1.94	0.79	+ 3.08 0.00 + 2.04 - 0.66	+ 6.29 +11.35 + 8.85 + 2.05	3.21 11.35 6.81 2.71		90 30 66 68	90 35 70 79
41	3.92	0.51	+ 6.46 + 9.77 - 2.41 + 7.53	+ 5.48 +12.34 + 4.63 + 7.30	2.57 7.04	0.98  0.23	62 56 49 75	60 57 49 69
42	0.92	5.66	+ 6.01 + 4.21	+12.24 +13.32	6.23 9.11		76 57	70 60

XI. APPENDIX (TABLE 2)—*Continued*

A	B	C	D	E	F.	G	H	I
NUMBER OF BEE	NORMAL BEE AV. <sup>o</sup> PER CM. IN RIGHT 957 MC. LIGHT	NORMAL BEE AV. <sup>o</sup> PER CM. TO LEFT 957 MC. LIGHT	ONE EYE BLACK AV. <sup>o</sup> PER CM. TURNED 24 MC. LIGHT	ONE EYE BLACK AV. <sup>o</sup> PER CM. TURNED 957 MC. LIGHT	+ VALUES OF E - D OR d	- VALUES OF E - D OR d	DURATION OF REC- ORDS IN SECONDS 24 MC. LIGHT	DURATION OF REC- ORDS IN SECONDS 957 MC. LIGHT
42			+ 8.71 + 8.45 + 9.49 + 7.73 + 9.03 +11.18 + 9.34	+11.20 +10.47 +12.47 +18.95 +13.70 +10.23 +13.48	2.49 2.02 2.98 11.22 4.67  4.14	     0.95	90 88 60 57 86 90 90	91 90 60 60 90 90 90
43	2.05 3.17		- 9.99 - 4.44 - 8.48 - 9.57	- 2.55 -12.61 - 8.43 -11.06	7.44  0.05	 8.17  1.49	60 76 90 70	57 90 106 60
44	0.00	0.57	+ 2.04 + 1.43 + 4.53 + 2.05	+16.10 +13.73 +10.36 + 8.76	14.06 12.30 5.83 6.71		61 60 73 72	76 45 70 70
45	5.20 8.01		+ 1.97 + 1.03 No rec- ord - 4.68	+ 2.39 + 3.80 -11.17 - 8.76	0.42 2.77	   4.08	48 62 59	60 69 84 60
51		3.41 3.75	- 4.49 - 3.96 + 2.87 - 8.30	+ 1.43 - 0.44 + 5.05 - 5.05	5.92 3.52 2.18 3.25		50 42 60 71	53 48 55 70
52	2.35	5.00	+ 6.22 + 2.37 + 3.30 + 8.44 + 6.94	+ 9.06 + 4.59 + 5.02 + 7.57 +11.07	2.84 2.22 1.72  4.13	   0.87	91 66 97 75 71	99 74 111 76 78
53		2.08 2.61	- 5.65 - 9.92 - 1.19 - 9.72	- 3.41 - 3.79 - 6.19 - 3.63	2.24 6.13  6.09	  5.00	59 73 55 30	65 59 50 32
54	6.91		- 0.79	+ 8.23	9.02		85	90



XI. APPENDIX (TABLE 2)—*Continued*

A	B	C	D	E	F	G	H	I
NUMBER OF BEE	NORMAL BEE AV. PER CM. TO RIGHT 957 MC. LIGHT	NORMAL BEE AV. PER CM. TO LEFT 957 MC. LIGHT	ONE EYE BLACK AV. PER CM. TURNED 24 MC. LIGHT	ONE EYE BLACK AV. PER CM. TURNED 957 MC. LIGHT	+ VALUES OF E - D or d	- VALUES OF E - D or d	DURATION OF REC- ORDS IN SECONDS 24 MC. LIGHT	DURATION OF REC- ORDS IN SECONDS 957 MC. LIGHT
54	3.11		+ 5.77 + 8.20 + 3.37 + 1.70	+ 8.25 + 6.63 + 3.54 + 3.71	2.48  0.17 2.01	 1.57	51 50 76 112	59 31 67 105
55		7.69 4.17	- 0.35 + 8.14 + 9.88 + 8.64	+ 4.36 + 8.89 + 7.89 + 6.78	4.71 0.75	 1.99 1.86	66 72 84 80	55 64 119 76
56		0.32 1.45	+ 1.50 + 1.14 - 3.73 -10.56	-12.86 -10.27 + 1.83 -18.47	  5.56	14.36 11.41  7.91	61 35 73 100	64 30 69 115
62	1.38	2.05	+ 1.55 + 4.54 + 1.88 + 4.42 +10.73 + 6.05 + 5.79 + 5.58 + 7.59 + 4.50	+ 1.36 + 2.98 + 9.18 + 8.71 +11.61 + 6.33 +10.79 + 5.87 + 7.45 + 4.47	  7.30 4.29 0.88 0.28 5.00 0.29	0.19 1.56     0.14 0.03	83 133 98 84 77 110 42 112 116 103	73 122 99 80 63 110 40 113 120 99
63	7.50	5.58	+ 0.48 + 3.94 +11.64 +10.69 + 4.13 + 3.04 + 3.59 + 0.18 + 2.99 + 7.00 +12.58 + 5.59	+11.83 + 8.56 + 2.61 + 8.45 + 4.50 + 3.41 +12.00 + 8.74 + 9.21 + 9.87 +14.74 + 7.79	11.35 4.62   0.37 0.37 8.41 8.56 6.22 2.87 2.16 2.20	  9.03 2.24	52 78 35 48 87 48 41 56 50 46 60 80	49 72 38 50 74 61 30 60 48 50 54 85
66		1.82	+ 6.78	+ 6.48		0.30	46	46

XI. APPENDIX (TABLE 2)—*Continued*

A	B	C	D	E	F	G	H	I
NUMBER OF BEE	NORMAL BEE AV. PER CM. TO RIGHT 957 MC. LIGHT	NORMAL BEE AV. PER CM. TO LEFT 957 MC. LIGHT	ONE EYE BLACK AV. PER CM. TURNED 24 MC. LIGHT	ONE EYE BLACK AV. PER CM. TURNED 957 MC. LIGHT	+ VALUES OF E - D OR d	- VALUES OF E - D OR d	DURATION OF REC- ORDS IN SECONDS 24 MC. LIGHT	DURATION OF REC- ORDS IN SECONDS 957 MC. LIGHT
66		0.18	+ 6.46	+ 4.07		2.39	85	93
			+ 1.73	+ 4.27	2.54		68	68
			+ 4.36	+ 8.27	3.91		54	55
			+ 7.40	+ 8.68	1.28		96	86
			+ 9.45	+ 5.87		3.58	60	60
			+ 5.23	+ 4.93		0.30	142	136
			+ 4.98	+ 4.60		0.38	100	104
			+ 3.02	+11.09	8.07		83	80
			+ 5.59	+10.32	4.73		101	122
68	6.08 1.94		+ 4.35	+13.12	8.77		97	94
			+11.65	+16.40	4.75		66	67
			+ 5.56	+14.91	9.35		54	59
			+ 8.97	+11.23	2.26		60	69
			+ 8.83	+ 9.82	0.99		87	100
			+10.57	+12.21	1.64		53	63
			+ 5.62	+12.06	6.44		75	60
			+10.48	+13.52	3.04		75	87
			+ 5.32	+11.79	6.47		91	99
			+ 4.37	+ 4.76	0.39		100	104
72	5.73 7.30		+ 7.13	+ 9.11	1.98		60	60
			+ 1.02	+24.35	23.33		20	35
			+ 2.65	- 0.29		2.94	77	58
			+ 0.87	+ 3.13	2.26		87	86
			- 3.92	- 0.66	3.26		103	100
			+ 6.77	+ 6.22		0.55	120	120
			+10.17	+12.94	2.77		100	91
73		4.94 2.24	+ 0.92	+ 8.27	7.35		39	40
			+ 1.53	+ 4.22	2.69		34	34
			- 1.42	+13.04	14.46		60	65
			+ 2.45	+10.89	8.44		100	100
			+ 2.00	+12.55	10.55		61	75
			+ 3.29	+13.79	10.50		93	101
			+ 2.88	+ 9.49	6.61		104	87
			- 0.12	- 8.89	9.01		147	147
			+ 6.07	+13.82	7.75		135	136
			- 1.69	+15.55	17.24		110	110

XI. APPENDIX (TABLE 2)—*Continued*

A	B	C	D	E	F	G	H	I
NUMBER OF BEE	NORMAL BEE AV. <sup>o</sup> PER CM. TO RIGHT 957 MC. LIGHT	NORMAL BEE AV. <sup>o</sup> PER CM. TO LEFT 957 MC. LIGHT	ONE EYE BLACK AV. <sup>o</sup> PER CM. TURNED 24 MC. LIGHT	ONE EYE BLACK AV. <sup>o</sup> PER CM. TURNED 957 MC. LIGHT	+ VALUES OF E - D or d	- VALUES OF E - D or d	DURATION OF REC- ORDS IN SECONDS 24 MC. LIGHT	DURATION OF REC- ORDS IN SECONDS 957 MC. LIGHT
77	4.22 7.46		+10.71	+14.43	3.72		57	75
			+ 9.62	+16.59	6.97		88	65
			+13.53	+18.39	4.86		48	40
			+12.43	+14.74	2.31		104	80
			+ 8.45	+14.92	6.47		90	80
			+11.64	+16.89	5.25		120	130
			+12.60	+15.38	2.78		75	70
			+15.29	+17.98	2.69		40	35
81	2.09 2.59		+11.57	+18.36	6.79		80	80
			+ 4.21	+10.60	6.39		114	117
			+ 1.03	+ 3.98	2.95		53	53
			+ 6.03	+ 7.78	1.75		116	106
			+ 5.76	+ 5.86	0.10		108	101
			+ 3.30	+ 8.35	5.05		70	65
			+ 4.59	+ 9.89	5.30		56	61
			+ 5.03	+ 7.49	2.46		63	63
82		5.81 0.65	+ 4.81	+13.52	8.71		57	80
			+ 2.76	+ 8.21	5.45		110	122
			+ 3.71	+12.60	8.89		67	67
			- 3.94	- 2.33	1.61		124	119
			- 5.37	- 5.01	0.36		110	123
			-10.30	- 6.43	3.87		60	60
			- 6.95	- 4.88	2.07		59	59
			- 2.59	+ 1.42	4.01		66	64
83	3.59 5.85		- 3.29	- 0.57	2.72		74	85
			- 7.06	+ 2.46	9.52		54	56
			- 6.40	- 5.29	1.11		70	80
			- 0.80	- 5.47		4.67	108	105
			+ 5.62	+15.93	10.31		81	76
			+ 8.37	+15.73	7.36		90	90
			+ 8.89	+10.53	1.64		104	104
			+ 6.93	+11.49	4.56		71	71
			+12.84	+21.53	8.69		58	60
			+ 9.39	+17.47	8.08		60	60
			+ 4.93	+16.39	11.46		94	90
			+ 4.53	+12.50	7.97		41	33
			+ 3.52	+14.42	10.90		133	128

XI. APPENDIX (TABLE 2)—*Continued*

A	B	C	D	E	F	G	H	I
NUMBER OF BEE	NORMAL BEE AV.° PER CM. TO RIGHT 957 MC. LIGHT	NORMAL BEE AV.° PER CM. TO LEFT 957 MC. LIGHT	ONE EYE BLACK AV.° PER CM. TURNED 24 MC. LIGHT	ONE EYE BLACK AV.° PER CM. TURNED 957 MC. LIGHT	+ VALUES OF E - D OR d	- VALUES OF E - D OR d	DURATION OF REC- ORDS IN SECONDS 24 MC. LIGHT	DURATION OF REC- ORDS IN SECONDS 957 MC. LIGHT
85		0.84 0.93	+ 6.46 + 1.41 + 7.08 + 6.87 + 4.34 + 1.73 + 4.61 + 6.13 + 4.68	+12.52 + 7.78 + 3.74 + 6.42 + 3.54 + 3.63 + 8.19 + 7.45 + 7.99	6.06 6.37	3.34 0.45 0.80	47 113 75 80 126 64 137 94 71	46 117 82 108 123 74 123 94 58
91	0.48 6.99		- 2.42 -10.05 - 6.44 - 7.33 + 2.18 + 0.73 +10.19	- 4.98 - 4.00 - 6.05 - 3.05 + 3.52 + 6.69 +27.54	6.05 0.39 4.28 1.34 5.96 17.35	2.56	125 85 95 81 110 64 30	125 80 95 81 131 64 30
92		10.66 8.67	+ 4.42 + 4.28 + 7.41 + 9.64 + 7.49 +14.19	+ 5.77 + 9.86 + 9.19 + 7.64 +17.34 +23.82	1.35 5.58 1.78	2.00	113 58 94 63 52 60	104 62 85 61 52 60
93	2.44	0.21	+ 1.61 + 0.19 + 2.87 + 2.37	+ 6.48 + 1.42 - 0.46 + 2.92	4.87 1.23 0.55	3.33	120 99 63 98	120 99 63 87
95		2.29 6.52	+ 2.14 + 1.13 0.00 + 0.32 + 1.29 + 0.93 + 2.39 + 3.67 + 0.85 + 0.21	+ 4.89 + 3.07 + 5.67 + 6.58 + 4.82 +11.94 + 7.18 + 7.11 + 6.35 + 1.93	2.75 1.94 5.67 6.26 3.53 11.01 4.79 3.44 5.50 1.72		118 100 58 71 70 34 69 66 82 61	123 109 46 80 78 30 74 67 75 60

XI. APPENDIX (TABLE 2)—*Continued*

A	B	C	D	E	F	G	H	I
NUMBER OF BEE	NORMAL BEE AV. ° PER CM. TO RIGHT 957 MC. LIGHT	NORMAL BEE AV. ° PER CM. TO LEFT 957 MC. LIGHT	ONE EYE BLACK AV. ° PER CM. TURNED 24 MC. LIGHT	ONE EYE BLACK AV. ° PER CM. TURNED 957 MC. LIGHT	+ VALUES OF E - D OR d	- VALUES OF E - D OR d	DURATION OF REC- ORDS IN SECONDS 24 MC. LIGHT	DURATION OF REC- ORDS IN SECONDS 957 MC. LIGHT
96	5.54	2.76	+10.46	+19.08	8.62	0.79	90	92
			+ 9.29	+ 8.50			195	192
			+11.89	+15.99	4.10		60	60
			+14.09	+17.84	3.75		60	60
			+ 5.33	+10.15	4.82		35	29
101		7.11 6.10	- 5.47	- 2.55	2.92	0.40	62	63
			- 1.67	- 2.07			88	79
			- 2.09	- 0.61	1.48		60	59
			- 1.02	- 1.00	0.02		67	70
			- 3.34	- 1.58	1.76		46	49
102	3.67 5.30		- 1.58	+14.37	15.95		49	49
			+ 2.98	+15.02	12.04		50	54
			+ 3.75	+11.08	7.33		71	78
			- 2.23	+ 1.45	3.68		62	66
			- 0.32	+ 6.46	6.78		64	53
			- 3.38	+11.11	14.49		57	58
			- 1.98	+ 4.52	6.50		41	60
			- 2.23	+10.19	12.42		88	88
			+ 3.55	+13.35	9.80		95	95
			- 1.28	+12.23	13.51		70	60
103	0.00 6.34		- 2.46	+13.48	15.94		57	57
			- 4.29	+11.25	15.54		77	60
			+ 0.16	+ 8.49	8.33		60	69
			- 1.26	+11.36	12.62		61	61
			- 0.44	+ 2.15	2.59		84	84
			- 3.75	+11.04	14.79		60	60
			+ 3.89	+ 6.47	2.58		60	65
			+ 2.27	+ 6.99	4.72		63	68
			- 0.18	+ 0.22	0.40		105	104
			+ 0.86	+ 4.22	3.36		62	64
105	5.93 8.81		+ 8.46	+14.64	6.18		68	60
			+ 9.19	+13.78	4.59		50	60
			+ 6.89	+14.10	7.21		80	80
			+ 6.55	+15.20	8.65		60	60
			+ 6.74	+14.83	8.09		93	93
			+ 6.21	+12.73	6.52		60	60
			+10.34	+16.05	5.71		60	60



XI. APPENDIX (TABLE 2)—*Continued*

A	B	C	D	E	F	G	H	I
NUMBER OF BEE	NORMAL BEE AV.° PER CM. TO RIGHT 957 MC. LIGHT	NORMAL BEE AV.° PER CM. TO LEFT 957 MC. LIGHT	ONE EYE BLACK AV.° PER CM. TURNED 24 MC. LIGHT	ONE EYE BLACK AV.° PER CM. TURNED 957 MC. LIGHT	+ VALUES OF E - D or d	- VALUES OF E - D or d	DURATION OF REC- ORDS IN SECONDS 24 MC. LIGHT	DURATION OF REC- ORDS IN SECONDS 957 MC. LIGHT
105			+10.03 + 9.04 + 7.46	+17.05 +11.01 +12.68	7.02 1.97 5.22		60 100 163	60 100 163
106	6.43 1.71		- 1.18 + 0.44 + 2.79 - 1.49 + 0.59 + 0.53	+ 1.49 + 3.22 + 6.72 + 5.19 - 5.49 + 5.33	2.67 2.78 3.93 6.68 4.90 4.80		70 81 41 35 77 103	68 79 41 35 77 115
121	1.61	6.87	+ 2.96 +11.57 + 3.13 + 1.91 - 4.71	+24.04 +19.71 +15.11 +12.12 +17.50	21.08 8.14 11.98 10.21 22.21		60 60 92 87 60	60 60 88 91 60
122	2.31 2.93		+ 1.88 + 3.65 + 3.25 - 4.39	+ 8.96 + 3.77 + 1.99 + 6.50	7.08 0.12  10.89	1.26	69 118 90 56	73 111 85 55
123		17.86 3.60	+ 7.16 + 1.79 -10.76 - 8.08 - 7.61 - 3.46 - 3.25 - 3.53 - 2.21	+18.55 - 1.21 + 0.59 + 2.29 - 2.14 + 1.12 + 5.69 + 0.56 + 1.97	11.39  11.35 10.37 5.47 4.58 8.94 4.09 4.18	4.00	54 65 69 79 60 140 60 58 90	60 69 67 79 60 135 58 58 101
124		2.59 3.00	+ 3.88 + 3.94 + 2.54 +10.36 +11.04 +10.39 + 5.81 + 6.55 + 7.67	+11.18 + 6.45 + 8.38 +11.19 +17.61 +17.41 +12.54 + 8.95 +10.19	7.30 2.51 5.84 0.83 6.57 7.02 6.73 2.40 2.52		73 53 79 80 90 60 58 74 60	68 59 71 80 90 60 58 60 60

XI. APPENDIX (TABLE 2)—*Continued*

A	B	C	D	E	F	G	H	I
NUMBER OF BEE	NORMAL BEE AV. PER CM. TO RIGHT 957 MC. LIGHT	NORMAL BEE AV. PER CM. TO LEFT 957 MC. LIGHT	ONE EYE BLACK AV. PER CM. TURNED 24 MC. LIGHT	ONE EYE BLACK AV. PER CM. TURNED 957 MC. LIGHT	+ VALUES OF E - D or d	- VALUES OF E - D or d	DURATION OF REC- ORDS IN SECONDS 24 MC. LIGHT	DURATION OF REC- ORDS IN SECONDS 957 MC. LIGHT
126		6.51	- 1.39	+ 3.11	4.50		105	100
		1.19	+ 4.51	- 2.89		7.40	48	50
			+ 3.73	+ 6.14	2.41		78	77
			- 1.44	+23.37	24.81		45	45
			+ 1.83	+ 4.41	2.58		107	107
			+ 4.57	+ 3.65		0.92	148	153
			+ 5.51	+ 4.32		1.19	72	72
			- 2.98	- 2.26	0.72		83	83
			- 1.49	+ 3.73	5.22		25	28
133	2.82 5.86		+ 2.39	+ 3.18	0.79		70	74
			+ 1.98	+ 3.26	1.28		69	68
			+ 1.54	+ 4.31	2.77		60	61
			+ 4.79	+ 9.29	4.50		60	60
			+ 1.76	+ 4.92	3.16		82	76
			+ 3.30	+11.54	8.24		38	40
			+ 3.35	+ 7.77	4.42		64	68
134		1.52	+ 8.29	+19.62	11.33		69	69
		1.22	+ 9.94	+19.60	9.66		57	57
			+ 5.67	+18.19	12.52		80	80
			+ 0.22	+18.93	18.71		60	60
			+ 3.83	+12.53	8.70		58	58
135		10.77	- 3.25	- 0.54	2.71		63	61
		10.88	+ 0.74	+ 2.03	1.29		95	89
			+ 1.29	+ 3.35	2.06		109	109
			- 9.49	- 5.85	3.64		58	59
			+ 2.28	- 1.37		4.65	71	71
			- 1.05	- 3.03		1.98	94	94
			- 0.33	- 7.67		7.34	79	76
			+ 0.44	- 6.66		7.10	58	59
137		5.99	+11.73	+19.09	17.36		60	60
		2.94	+15.82	+16.09	0.27		49	49
			+10.59	+16.45	5.86		70	70
			+15.78	+16.57	0.79		60	60
			+14.31	+15.40	1.09		78	78
			+14.71	+22.57	7.86		60	60
			+14.69	+17.02	2.33		70	70

XI. APPENDIX (TABLE 2)—*Concluded*

A	B	C	D	E	F	G	H	I
NUMBER OF BEE	NORMAL BEE AV. ° PER CM. TO RIGHT 957 MC. LIGHT	NORMAL BEE AV. ° PER CM. TO LEFT 957 MC. LIGHT	ONE EYE BLACK AV. ° PER CM. TURNED 24 MC. LIGHT	ONE EYE BLACK AV. ° PER CM. TURNED 957 MC. LIGHT	+ VALUES OF E - D OR D	- VALUES OF E - D OR D	DURATION OF REC- ORDS IN SECONDS 24 MC. LIGHT	DURATION OF REC- ORDS IN SECONDS 957 MC. LIGHT
137			+13.38 + 8.43 +15.92	+15.64 +16.71 +18.08	2.26 8.28 2.16		60 60 60	60 60 60
138		6.55 8.37	- 0.18 - 0.37 - 1.12 - 9.00 - 3.07 - 1.97 - 6.19	- 4.67 + 0.13 +13.52 - 3.52 + 1.60 + 2.57 - 1.08		4.49	129 62 65 70 115 77 69	129 58 60 70 115 74 69

Resumen por el autor, R. W. Hegner.  
Universidad John Hopkins.

Los efectos de los factores ambientes sobre los caracteres hereditarios de *Arcella dentata* y *A. polypora*.

El protozooario rizópodo *Arcella dentata* ha sido sometido por el autor a la influencia de varios factores ambientes. Ejemplares de tamaño y número de espinas conocidos han sido tratados con soluciones de silicato sódico y alcohol etílico; también fueron sometidos a varias temperaturas y nutridos insuficientemente. En los descendientes de animales así tratados aparecen cambios en el diámetro de la concha y en el número, tamaño y forma de las espinas, pero recobran la condición normal cuando se crían de nuevo bajo condiciones normales.

Translation by José F. Nonidez  
Carnegie Institution of Washington

## THE EFFECTS OF ENVIRONMENTAL FACTORS UPON THE HERITABLE CHARACTERISTICS OF ARCELLA DENTATA AND A. POLYPORA<sup>1</sup>

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SEVEN FIGURES

While carrying on a series of experiments for the purpose of testing the efficacy of selection as a means of isolating heritably diverse lines within a clone of *Arcella dentata*, and later while studying the nucleocytoplasmic relations in this species and in *A. polypora*, several experiments were performed with the purpose of determining the effects of environmental factors upon the heritable characteristics of these organisms.

If it is possible to modify organisms by means of external factors in such a way that the diversities produced will persist after the disturbing factors have been eliminated, we may account for the numerous heritable diversities that have been described among the lower organisms by the presence of such factors in their environment.

### UNDERFEEDING

During the selection work on *Arcella dentata* (Hegner, '19) the organisms were supplied with an abundance of organic matter shaken from the leaves and stems of aquatic plants. The pond water in which this food material was suspended was then diluted with distilled water. At first the number of spines was used for purposes of selection, but later the diameter of the shell was also employed, since the organisms were found to be remarkably constant in size, and diameter of shell and spine

<sup>1</sup> The studies presented in this paper are incomplete, but they are published at this time, since the writer will probably be unable to carry on further experimental work with *Arcella* in the near future.



number were shown to be closely correlated. It was also discovered that a definite relation exists in these animals between nuclear number and size and between chromatin mass and cytoplasmic mass. These facts led to the experiments on underfeeding described below. The data obtained furnish information regarding fission rate and variations in diameter of the shell.

*a. The effects of underfeeding upon the rate of fission*

Specimens of *Arcella dentata* were taken from cultures that were being used for selection experiments, and placed in a medium consisting of one-half distilled water and one-half filtered pond water. Instead of a gradual decrease in nuclear and cytoplasmic material and the cessation of reproduction as was expected, several specimens proceeded to divide, and within a month three rather large families had been produced by them. Evidently, even after being filtered, enough food remained in the pond water to enable the arcellas to grow and reproduce. However, the amount of food present was much less than was ordinarily given to the specimens in laboratory cultures, and several interesting results appeared which were apparently due to underfeeding.

There was a marked retardation of the division rate during the period when the specimens were underfed. Fission occurred at intervals of from two to ten days, with the following means:

Family 2. Average interval between fissions, 5.25 days.

Family 3. Average interval between fissions, 3.47 days.

Family 4. Average interval between fissions, 4.15 days.

In contrast to this, the division rate of the parental lines under normal cultural conditions was approximately 2.50 days. Previous work on pure lines in *Arcella dentata* indicated that different lines differ in division rate, but these were all being reared under similar conditions and supplied with an abundance of food. The differences in division rate between these pure lines were, therefore, not due to differences in nutrition. The results just described, however, show that it is necessary to keep

food conditions constant when undertaking experiments involving division rate. They also prove that *Arcella dentata* is able to grow and reproduce under adverse conditions with respect to the food supply, but at a much less rapid rate.

*b. The effects of underfeeding upon the diameter of the shell*

The offspring of parents that were underfed showed the effects of this treatment immediately, being smaller than their parents in every case. When these offspring were underfed, they likewise gave rise to smaller offspring than the normal for the line, but not, on the average, smaller than themselves. When, on the other hand, these small offspring of underfed parents were

TABLE 1

*Arcella dentata*. Table showing the distribution in diameters (in units of  $4.3 \mu$ ) of specimens in families, 2, 3, and 4 during periods when they were being reared in normal medium and when they were underfed

	DIAMETER														
	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Family 2. Normal.....						2	6	2	1						
Family 2. Underfed.....	1		4	1	3	2	1								
Family 3. Normal.....												2	1	1	2
Family 3. Underfed.....								3	1	11	7	9	4	1	
Family 4. Normal.....												2	1	2	
Family 4. Underfed.....								1	4	15	8	4	2		

returned to normal cultural conditions, their first offspring showed the effects of the abundance of food, becoming close to the normal. Also, when full-sized specimens that were produced under normal conditions and which had given rise to small offspring when subjected to underfeeding, were again supplied with an abundance of food, the size of their offspring immediately attained that normal for the line. The six cases on following page present data characteristic of the series.

Table 1 gives the distribution of the diameters of the specimens reared in families 2, 3, and 4, both when the parents were in normal cultural conditions and when underfed. The following means bring out the significance of these data:

Table of means

	NUMBER OF SPECIMENS	MEAN SPINE NUMBER	MEAN DIAMETER
Family 2. Offspring produced while parent in normal condition.....	11	11.50	27.18
Family 2. Offspring produced while parent underfed.....	12	10.82	24.25
Family 3. Offspring produced while parent in normal condition.....	6	14.17	33.50
Family 3. Offspring produced while parent underfed.....	36	13.54	30.94
Family 4. Offspring produced while parent in normal condition.....	15	13.20	33.00
Family 4. Offspring produced while parent underfed.....	24	12.88	30.47

Six cases

	SPINE NUMBER	DIAMETER
Family 2. Original progenitor.....	10	27
First offspring while parent underfed.....	9	23
First offspring when parent returned to normal.....	11	27
First offspring when parent again underfed.....	10	26
Second offspring when parent still underfed.....	9	24
Family 2. First offspring original progenitor.....	9	23
First offspring while parent underfed.....	11	23
Second offspring while parent underfed.....	11	25
First offspring when parent returned to normal.....	11	26
Family 2. First offspring of first offspring.....	11	23
First offspring while parent underfed.....	10	21
First offspring when parent returned to normal.....	10	26
Family 3. Original progenitor.....	15	35
First offspring while parent underfed.....	14	31
Second offspring while parent underfed.....	14	30
Family 3. First offspring of original progenitor.....	14	31
Fourth offspring while parent underfed.....	12	28
Sixth offspring when parent returned to normal.....	14	33
Family 4. Original progenitor.....	14	32
Fourth offspring while parent underfed.....	11	29
First offspring when preceding specimen placed in normal...	14	33
First offspring when preceding specimen underfed.....	14	29
First offspring when preceding specimen underfed.....	12	30

The mean diameter of the line from which the progenitor of family 2 was taken was 26.40 units and the mean spine number 10.90. The mean diameter of the line from which the progenitors of families 3 and 4 were taken was 34.00 units and the mean spine number 14.59.

In connection with these experiments in underfeeding it may be noted that a large majority of wild specimens, when collected from the vegetation in ponds, possess very little cytoplasm. This is probably due to the struggle these minute organisms must undergo in their natural habitat. On the other hand, it is not unusual after being brought into the laboratory for the offspring of such wild specimens to average much smaller than their original progenitors, although they are supplied with an abundance of food. Thus, in *Arcella polypora* the progenitors of twenty-six families ranged in diameter from 23 to 35 units of  $4.3\ \mu$  each, with a mean diameter of 30.42 units, whereas their first offspring ranged in diameter from 21 to 32 units with a mean diameter of 27.50 units. No definite reason can be given for this decrease in size under laboratory conditions, but perhaps the abundant food supply is responsible, resulting in the initiation of fission before the cytoplasm has increased to the amount normally present when the animals are in their natural habitat.

The results of these experiments prove that size and spine number in *Arcella dentata* are affected by the food supply. Selection experiments involving these characteristics in this organism and probably in other similar organisms, as well, must therefore be carried out so as to provide a constant food supply.

This factor, however, was carefully controlled in the selection experiments that have been reported on *Diffugia* (Jennings, '16), *Centropyxis* (Root, '18), and *Arcella* (Hegner, '19 a).

#### THE ADDITION OF SODIUM SILICATE

The fact that Whitney ('16) caused the transformation of the rotifer *Brachionus pala* into the variation *Brachionus ampiceros* by the simple addition of sodium silicate to the medium in which they were reared, led to the use of this substance in experiments on *Arcella dentata*. It seemed probable that the presence of an

excess of sodium silicate might facilitate shell production and bring about the formation of variations, such as longer spines. The method employed was to make up daily or every other day culture media as usual and then add one drop of sodium silicate to 100 cc. of the medium. Solutions of greater strength were tried, but the organisms did not thrive in them.

The experiments were begun with thirty specimens taken from the lines that were being used at the time for selection experiments. From these, five families were reared as follows:

Family 14 with 135 specimens

Family 15 with 20 specimens

Family 20 with 11 specimens

Family 25 with 9 specimens

Family 26 with 19 specimens

No difficulty was experienced in obtaining large families, the number of specimens recorded being limited only by the amount of time available to care for them.

*a. Fission rate*

The rate of fission of specimens grown in the sodium silicate medium decreased immediately from an average of one division in 2.50 days to one division in about four days. Evidently the presence of sodium silicate affected adversely the food material upon which the Arcellas were feeding or else hindered the feeding process or the rate of digestion and assimilation.

*b. Size and spine number*

Instead of an increase in spine number and length and in the size of the shell as was expected, the immediate result of the changed medium was a decrease in all these characters. The pedigrees shown in figures 1, 2, and 3 illustrate the differences in diameter and spine number very clearly. Many of the specimens were badly crinkled, which, no doubt, accounts in part for their smaller diameter; in others the binucleate condition was lost and uninucleates appeared. Wherever this occurred a 1 in paren-



theses is added in the figures (figs. 1, 2, and 3). In another place (Hegner, '20) the writer has shown that this change from the binucleate to the uninucleate condition is accompanied by a decrease in size, as indicated also by these experiments. The

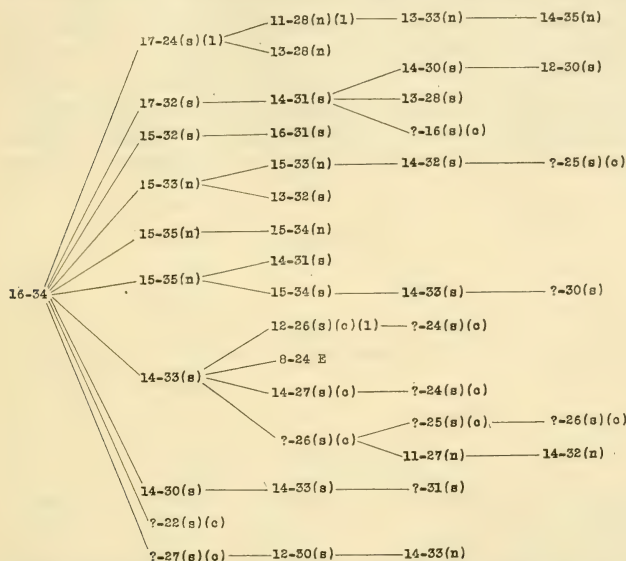


Fig. 1 *Arcella dentata*. Part of the pedigree of family 14. The numbers indicate the number of spines and the diameter in units of  $4.3 \mu$ . For example, the original progenitor (16-34) possessed sixteen spines and was 34 units in diameter. The letters and numbers in parentheses should be interpreted as follows: (s) = specimen produced while parent was in sodium silicate solution; (n) = specimen produced while parent was in normal medium; (c) = specimen with crinkled shell; (l) = specimen with only one nucleus; (?) = indeterminate number of spines.

outlines in figures 4 and 5 show the decrease in the length of the spines. In many cases the spines did not extend beyond the edge of the shell, being represented only by ridges on the dorsal surface of the shell. The experiments extended over the period from March 18 to May 15, 1918. The largest family, no. 14

(fig. 1), was begun on March 21, and its original progenitor lived throughout the rest of the period, giving rise in that time to ten offspring. Specimens from branches of this family that had been constantly subjected to the sodium silicate solution were at intervals transferred to the normal culture medium, but in every case the size, spine number, and spine length characteristic of the parent line were immediately regained. Family 14 was continued until it contained 135 members and included specimens of the eighteenth generation, but no heritable variations were noted that could be attributed to the changed medium. Figures 4 and 5 illustrate the changed condition of the offspring

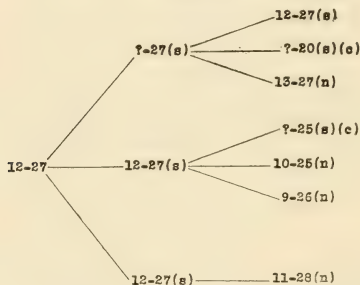


Fig. 2 *Arcella dentata*. Part of the pedigree of family 15. For description see figure 1.

when the parents of this family and of family 15 were subjected first to the sodium silicate solution and later were replaced in the normal medium.

### *c. Color of the shell*

Another character that was modified by the presence of sodium silicate was the color of the shell. Arcellas that are reared in a normal medium have a shell that is at first almost transparent, but gradually changes to a pale yellow, and in time becomes a very deep brown. The offspring reared from parents that were kept in the sodium silicate solution were pale yellowish green, in color, as long as they remained in this medium, but became of the

normal brown color as soon as they were transferred to normal cultures. The color mechanism of *Arcella* seems to be very sensitive to environmental factors. During the work on selection it became evident that pure lines probably exist with respect to the length of time it requires for the young to reach their definitive color and also with regard to the intensity of the color attained. Changes in the character of the food were likewise found to affect the color. For example, when food was obtained from vegetation taken from a cement tank in the botanical garden on the Johns Hopkins University campus, the offspring became dark brown almost immediately after fission; specimens reared

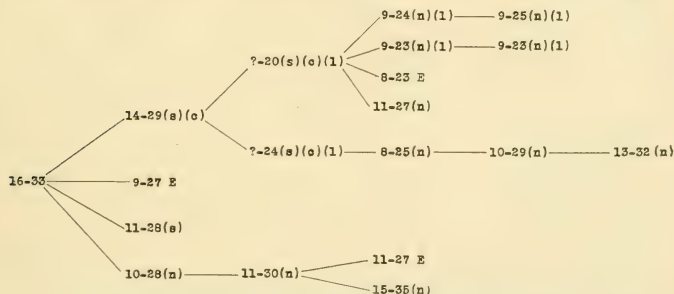


Fig. 3 *Arcella dentata*. Complete pedigree of family 26. For description see figure 1.

in hay infusions were more yellowish in color than normal, and those fed on material collected from a spring-water fish pond at Cold Spring Harbor, Long Island, were characteristically pale.

*Arcella* thus resembles the many other organisms that are modified by changes in the environment; they remain so as long as they are in this environment, but return to their former condition when transferred back to the original medium.

#### THE ADDITION OF ALCOHOL

One of the substances to which Protozoa have been found to be resistant is alcohol. Experiments were begun to determine the effects of alcohol on *Arcella dentata*, but were terminated

because of lack of time to keep them going. Sufficient data were obtained, however, to prove that these organisms are able to live and reproduce in a medium containing from 0.25 to 1 per cent of alcohol. Three offspring were obtained from one of the

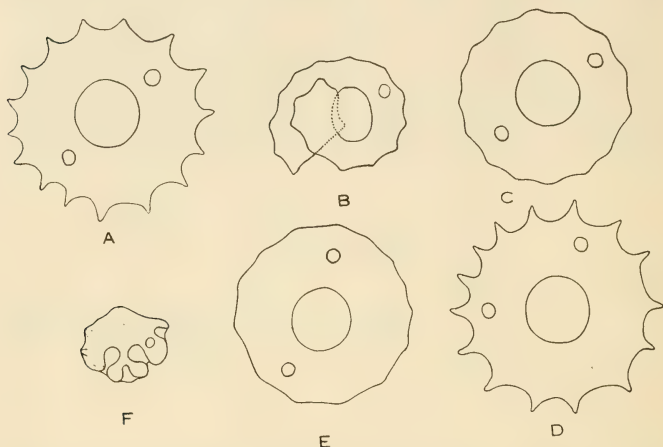


Fig. 4 *Arcella dentata*. Family 14. Camera-lucida sketches ( $\times 207$ ) showing:

A = the original progenitor; with sixteen spines and diameter of 34 units.

B = the first offspring after A was placed in a solution of one drop of sodium silicate to 50 cc. of normal medium.

C = the third offspring of A, produced while A was in a solution of one drop of sodium silicate to 100 cc. of normal medium.

D = fourth offspring of A, produced immediately after A was transferred from the sodium silicate solution back to normal medium.

E = seventh offspring of A, produced immediately after A was transferred from normal medium to sodium silicate solution.

F = a specimen of the seventh generation after continuous subjection to sodium silicate.

specimens that was kept in a 0.50 per cent solution of alcohol; two of these and the parent were still alive thirty-five days after the experiment was begun. The rate of fission was very slow, probably because of the effects of the alcohol upon the food.

One of the progeny was irregular in shape and another was without definite spines. It is evident from the results that alcohol is injurious to these organisms, although they are able to withstand the presence of a considerable amount for a long period.

#### TEMPERATURE

A number of specimens of *Arcella dentata* were collected on December 27, 1917, from pond weeds under a layer of ice eight inches thick. Many of these had no well-developed spines, but

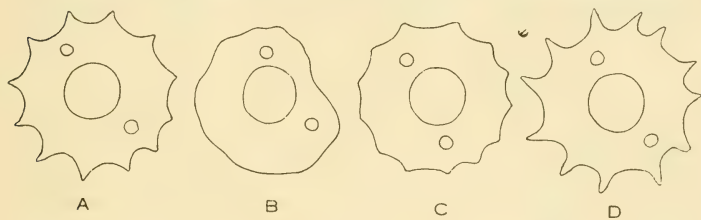


Fig. 5 *Arcella dentata*. Family 15. Camera-lucida sketches ( $\times 207$ ) showing:

A = the original progenitor with twelve spines and diameter of 27 units.

B = the first offspring after A was placed in a solution of one drop of sodium silicate to 100 cc. of normal medium.

C = the first offspring of B, after B had produced two offsprings while in sodium silicate solution and was then transferred to normal medium.

D = the first offspring of C while C was in normal medium.

their progeny, when reared under laboratory conditions, exhibited a complete set of fully formed spines. This suggested that perhaps the low temperature might have been responsible for the lack of spines in the 'wild' parents. Several experiments were begun to test this hypothesis, and although they were not extensive they indicated that length of spine and temperature may be correlated since, as shown in figure 6, the spines of offspring reared at a temperature of about  $10^{\circ}\text{C}$ . are smaller than those of their parents which were reared at room temperature.



## SPECIMENS WITH BENT, OVAL SHELLS

Among the specimens of *Arcella* polypora that were collected at Cold Spring Harbor during the summer of 1918 were many with a shell that was oval in outline and contained an oval mouth opening and at the same time was bent slightly as indicated in figure 7. Most of these were obtained from duck weed taken from a small pond that was gradually drying up, but others were found in a small lake. Leidy ('79, pl. XXVIII, figs. 36 and 37) figures shells similar to these. A number of specimens were isolated and their progeny examined. The offspring of the first generation were either circular in outline or nearly so and almost

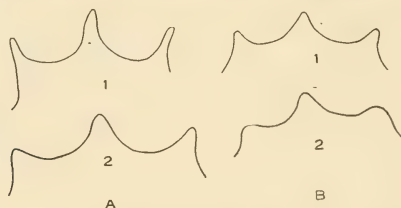


Fig. 6 *Arcella dentata*. Camera-lucida sketches ( $\times 450$ ) of three of the spines belonging, respectively, to normal specimens (A, 1 and B, 1) and to the first offspring produced by them when subjected to low temperature (A, 2 and B, 2).

flat, and those of later generations were entirely normal when reared under laboratory conditions. A few measurements are given below.

DIAMETER OF PARENT	DIAMETER OF FIRST OFFSPRING	SHAPE OF FIRST OFFSPRING
28 x 25	25	Flat, almost circular
29 x 25	27	Almost flat, almost circular
28 x 25	29	Almost flat, almost circular

A large number of specimens were placed in Syracuse watch-glasses and examined every day for a week. Many offspring were produced by them, but in every case they were quite normal or nearly so. Since the descendants of these bent, oval speci-

mens were normal and since no specimens of this sort have appeared among the thousands reared in the laboratory, it seems safe to conclude that some environmental condition is responsible for this peculiarity and that as soon as the controlling factor is removed, the normal characteristics are regained.

#### SUMMARY AND CONCLUSIONS

*a.* When specimens of *Arcella dentata* are underfed, the interval between successive divisions increases from an average period of 2.50 days to a period of about 4 days; the shell decreases in diameter on the average 2.68 units of  $4.3\ \mu$  each, and the spine

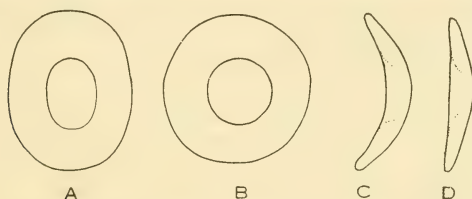


Fig. 7 *Arcella polypora*. Camera-lucida sketches ( $\times 207$ ). A = dorsal view of 'wild' specimen with oval shell and mouth. C = side view of same showing bent condition of the shell. B = dorsal view of first offspring of A under laboratory conditions. D = side view of B.

number also decreases slightly. The offspring of underfed parents produce progeny normal in size and spine number when given an abundance of food, and parents that have been underfed likewise give rise to normal offspring under similar conditions. Wild specimens are often poor in cytoplasmic content, but their offspring are frequently smaller than the parents when reared under laboratory conditions.

*b.* *Arcella dentata* will grow and reproduce in a medium containing one drop of sodium silicate to 100 cc. of water. The fission rate decreases, the average interval between fissions increasing from 2.50 to 4 days. The size of specimens produced while the parents are in the sodium silicate solution is less than that of progeny formed under normal conditions. The most con-

spicuous change brought about by the presence of sodium silicate is the almost complete loss of spines. The color of the shell, which becomes brown in a normal medium remains a pale greenish yellow in a sodium silicate solution. Specimens reared in sodium silicate and then returned to a normal medium regain the fission rate, size, spine length, and color or characteristic of the race.

c. Specimens of *Arcella dentata* are able to grow and reproduce in a medium containing from 0.25 to 1 per cent of alcohol. Alcohol, however, is shown to be injurious, as indicated by the retarded fission rate and irregularities in the shells of the offspring.

d. Several experiments seem to indicate that temperature influences the length of the spines of *Arcella dentata*, and that the lower the temperature the smaller the spines become.

e. Wild specimens of *Arcella polypora* that possessed a bent, oval shell with an oval mouth opening gave rise under laboratory conditions to offspring with a flat circular shell and a circular mouth opening. The bent, oval condition is probably due to an unknown environmental factor.

f. The environmental factors to which specimens of *Arcella* have been subjected cause distinct variations from the normal racial conditions, but these modifications persist only so long as the modifying factors are operative. No heritable diversities were observed that were due to the changed conditions. The experiments bring out no data that affect the results obtained by Jennings, Root, and the writer in isolating heritably diverse lines within families of fresh-water rhizopods during vegetative reproduction.

## LITERATURE CITED

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Resumen por la autora, Mary Elizabeth Collett.  
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La toxicidad de los ácidos sobre los infusorios ciliados.

La autora ha determinado la toxicidad del ácido clorhídrico y la de unos 15 ácidos orgánicos en varias concentraciones y temperaturas sobre *Paramoecium caudatum*, *Stylonichia pustulata*, *Euplotes patella* y *Vorticella nebulifera*. En concentraciones de 0.001 y 0.00008 N el orden tóxico es groseramente paralelo al orden de disociación, indicando esto que el ión H es un factor importante en la toxicidad. El orden tóxico varía algo con la temperatura, concentración y con la especie. Si se disminuye la ionización (como sucede con las mezclas de ácido clorhídrico con un ácido débil) algunos de los ácidos son menos efectivos, indicando esto que el anión es tóxico, así como el ión H. Los coeficientes de temperatura para intervalos de diez grados entre los 10° y 33°C indican que tanto las reacciones químicas como las físicas tienen influencia en la acción tóxica. Los ácidos acético y butírico son irregulares en lo referente a su toxicidad, puesto que esta función en *Euplotes*, aunque no en *Paramoecium*, aumenta con temperaturas inferiores y también superiores a los 20°.

Translation by José F. Nonidez  
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## THE TOXICITY OF ACIDS TO CILIATE INFUSORIA

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SIX GRAPHS

### INTRODUCTION

Many workers have studied the toxic effect of acids and have tried to arrive at an explanation of their mode of action. Very few of the experiments, however, are quite satisfactory. One difficulty lies in the choice of material: for example, in studies of the effect upon dogs of the feeding (Walter, '77) or intravenous injections of acids (Szili, '09) or the effect upon fish or tadpoles of adding acids to the external medium (Loeb and Wasteneys, '12; Roaf-Whitley, '04; Unger, '16; Winogradoff, '11), the material is too complex for a ready analysis of results. Some of the best work of the sort is that of Winogradoff ('11), who watched the process of injury to heart, corpuscles, muscle, etc., in small transparent tadpoles. More satisfactory material has been found in tissue or isolated cells, such as rootlets, muscle, ciliated cells, and erythrocytes (Kahlenberg-True, '96; Loeb, '98; Harvey, '14; Landsteiner and Prasek, '13) where conditions are much less complex. Aside from obvious crudities, such as not guarding against evaporation or not indicating time accurately, the experiments have most often been unsatisfactory because of lack of data for more than one concentration of the acids used or because of faulty temperature control. The usual method has been to determine upon some one tissue the toxic order of a series of acids, all at the same concentration (or at the concentration necessary to kill in a fixed time) and then to try to explain their relative toxicity by correlating the results of this one experiment with the physical properties of the acids.

This is not a satisfactory procedure, as was pointed out by Crozier in his work upon permeability. Lack of uniformity in concentration and temperature makes a comparison of different experiments almost impossible, even when the reactions involved are the same, and there are relatively few experiments in which several kinds of material or several physiological processes have been studied all together. The aim of the present experiments is to determine the relative toxicity of a series of acids, at several concentrations each and at various temperatures, to different sorts of nearly related material, and from these more complete experiments to draw what conclusions are possible as to the nature of the toxic action. I am greatly indebted to Dr. M. H. Jacobs, who suggested the problem, for helpful advice and criticism.

#### METHOD

The organisms observed in the following experiments were *Paramoecium caudatum*, *Styloichia pustulata*, *Euplotes patella*, and *Vorticella nebulifera*. They were grown in small jars of boiled hay infusion made with pond-water and seeded with decaying leaves, etc. Though many forms appeared in the cultures, only these four were constantly present in sufficient numbers to be useful for a long series of experiments.

The acids used were hydrochloric, formic, acetic, propionic, butyric, valeric, lactic, oxalic, malonic, tartaric, citric, benzoic, salicylic, and phthalic. All were made up at 0.1 N and titrated with litmus against 0.1 N NaOH. These stock solutions, as well as the weaker solutions made from them for use in the experiments, were kept in paraffined bottles, for they deteriorate rapidly in unprotected reagent bottles, even those prepared by thorough steaming.

The organisms were always washed overnight before use in order to eliminate fluctuations due to the changing composition of the culture medium. They were collected from the culture in as concentrated masses as possible and pipetted into about twenty times their volume of fresh pond-water. After twelve hours' washing they were concentrated again by means of a

hand centrifuge. This treatment does not injure them. If they are washed less than five hours, the results are apt to be irregular, but five hours seems to be almost as satisfactory as a longer period. Pond-water was used because ordinary distilled water seems to be somewhat injurious. After being washed and reconcentrated, the organisms were transferred to a watch-glass, in which they could be kept in apparently normal condition for several hours.

In order to test their resistance to the acids, the following procedure was adopted. A test-tube was filled with about 15 cc. of the solution to be tested, and less than 0.1 cc. of the concentrated culture was introduced with a capillary pipette. After being inverted once or twice for mixing, the test-tube was corked and placed in a water-bath, where the temperature could be held at the desired level throughout the experiment. Samples were poured at intervals into watch-glasses and observed under the low power of the compound microscope. It was found desirable to use watch-glasses with curved rather than vertical sides in order to prevent collection of the organisms at the margin where they are difficult to see. It is not enough to note the time at which the organisms cease to move about, for the cilia usually beat for some time afterward. Since there is often considerable variation in individual resistance, the time at which the cilia of just over half cease to beat was adopted as a better measure of average resistance than the time at which all are dead. Every solution was tried five or even ten times over, usually on different days, in order to check deviations due to biological differences, which, however, were relatively slight. In the following tables the averages of all the determinations are given.

#### DISCUSSION OF RESULTS

##### *A. Concentration*

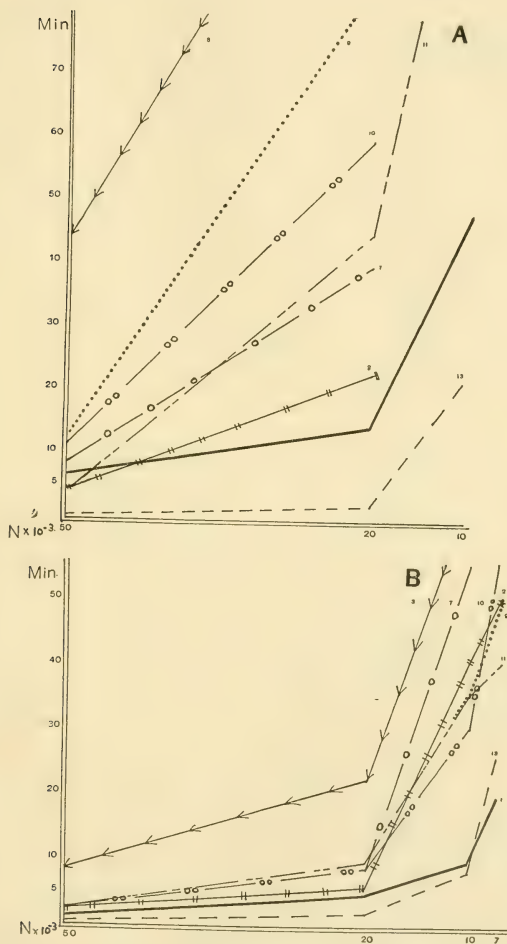
In these experiments each acid of the series was used at four of the following concentrations, viz., 0.001, 0.0005, 0.0002, 0.0001, 0.00008 N. Full data on the effects of these solutions are given in table 1, but the important points are perhaps

clearer in graph 1. It will be observed that for *Paramoecium* at 20° salicylic and HCl are at all concentrations the most toxic of the acids studied and acetic and butyric the least so, with the other acids scattered in between. But although in a general way the toxic order is similar at the different concentrations, there are several important exceptions. For example, the toxic order at 0.0002 N is formic > tartaric > oxalic = malonic, while at 0.0001 N the order is tartaric > malonic > formic > oxalic. This change in order is indicated by intersection of the curves. There is a similar though less important change at the next concentration, where the curve for HCl crosses that of salicylic and the tartaric curve crosses those of malonic and formic. With *Euplotes* change in concentration produces less variation in order, but instead a considerable variation in the degree of difference existing between the acids. Thus at 0.0005 N HCl formic and benzoic acids kill in 1½, 2½, and 3 minutes, respectively, while at 0.0001 N the relative times are 9, 40, and 35 minutes. Obviously, if conclusions were based solely upon the results for a single concentration, 0.0005 N for instance, they would not be valid, since the results at other concentrations are in so many respects different.

This conclusion agrees with the results obtained by Crozier ('16) in studying the rate of penetration of acids into the mantle cells of *Stichopus*, which contain a natural indicator. He found that the relative order obtaining at a concentration of 0.01 N was in many respects unlike the order at 0.001 N, and that the order of penetration found by Harvey ('14) and by Haas ('16) at 0.01 N with different material closely resembled his results at 0.01 N, but not his results at other concentrations. If other

## KEY TO GRAPH SYMBOLS

- |                        |                       |
|------------------------|-----------------------|
| 1. Hydrochloric ———    | 8. Citric ——— ——      |
| 2. Formic — — —— — —   | 9. Malonic .....o     |
| 3. Acetic —<——<——      | 10. Tartaric ———o o—— |
| 4. Propionic —< ——< —— | 11. Benzoic ———:——    |
| 5. Butyric ———<——<——   | 12. Phthalic ———:——   |
| 6. Lactic ———o——       | 13. Salicylic ———:——  |
| 7. Oxalic ———o——       |                       |



Graph 1, A, B Toxicity of equinormal acids at 20°C. (Euplotes above, Paramoecium below.) Should be  $N^{-5}$  and not  $N^{-3}$ .



TABLE 1

*Effect of temperature on toxicity of equinormal acids. Time in minutes*

	N/	HCl	OXALIC	TARTARIC	MALONIC	FORMIC	ACETIC <sup>d</sup>	BUTYRIC <sup>d</sup>	BENZOIC	SALICYLIC
<i>Paramoecium</i>										
12° .....	0.001						10	11		
	0.0005	5	8	7	7	4	17	17	7	< $\frac{1}{2}$
	0.0002	10	16	17	25	15	50	60	30	3
	0.0001	22		40	120	38			75	14
	0.00008									
15° .....	0.001						6	6 $\frac{1}{2}$		
	0.0005	4	4 $\frac{1}{2}$	5	7	4	12	14	5	
	0.0002	6	12 $\frac{1}{2}$	14	25	9	31	43	20	
	0.0001	15		35	100	40			70	10
	0.00008									48
20° .....	0.001						4	6		
	0.0005	1 $\frac{1}{2}$	3	3	3 $\frac{1}{2}$	2 $\frac{1}{2}$	8	9	3	< $\frac{1}{2}$
	0.0002	4 $\frac{1}{2}$	9	9	10	6	22	27	10	2
	0.0001	9	55	30	35	40		60	35	7 $\frac{1}{2}$
	0.00008	18		60	50	50			40	25
25° .....	0.0002			6	9	5			8	
	0.0001	6		18	28	20			15	6
	0.00008	10				22			17	
30° .....	0.0002	3		3 $\frac{1}{2}$	11	3 $\frac{1}{2}$			5	
	0.0001	5		7	20	14			9	3
	0.00008	8		10		20			14	6 $\frac{1}{2}$
<i>Styloichia</i>										
20° .....	0.001						2			
	0.0005	1 $\frac{1}{2}$	3 $\frac{1}{2}$	3 $\frac{1}{4}$	3 $\frac{1}{2}$	2 $\frac{1}{2}$	4	8	2 $\frac{1}{2}$	< $\frac{1}{2}$
	0.0002	5	11	8	11	7	24	35	10	1 $\frac{1}{4}$
	0.0001	9 $\frac{1}{2}$		50	90	40			35	6 $\frac{1}{2}$
	0.00008	20						60	60	25
<i>Euplotes</i>										
12° .....	0.001						7	9		
	0.0005	12	10	15	18	12	25	33	8	$\frac{1}{2}$
	0.0002	22	40	60	100	25			45	3
	0.0001	43				100+			100	22

TABLE 1—Continued

	N/	HCl	OXALIC	TARTARIC	MALONIC	FORMIC	ACETIC <sup>1</sup>	BUTYRIC <sup>1</sup>	BENZOIC	SALICYLIC
15°.....	0.001						10			
	0.0005	11		12	17	9	20	35	8	< $\frac{1}{2}$
	0.0002	14		60		15			50	
	0.0001	45								21
20°.....	0.001						17	25		
	0.0005	7	9	12	13	5	45	55	5	< $\frac{1}{2}$
	0.0002	15	40	60	85	23	100+		45	2 $\frac{1}{2}$
	0.0001	45							120	22
	0.00008	80								90+
25°.....	0.0005			9						
	0.0002			30	75	15			38	
	0.0001	30				70				22
30°.....	0.001						6	8		11
	0.0005		7	6	8		20	25		
	0.0002	8		30	50	10			15	
	0.0001	20				70			55	
	0.00008	43								

<sup>1</sup> Acetic and butyric were tested at 10° and not at 12°.

properties of a series of acids are similarly varied with dilution, as my experiments indicate, then very few of the experiments so far available can be fairly compared, since most observers report only one concentration, which may lie anywhere between 1 and 0.00001 N.

### B. Specific differences

In order to study specific differences adequately, precautions must be taken to keep all the material under identical conditions. Uniformity was insured in the present experiments by using a mixed culture and testing the organisms all together. Graph 2 shows typical results; full data are given in table 1.

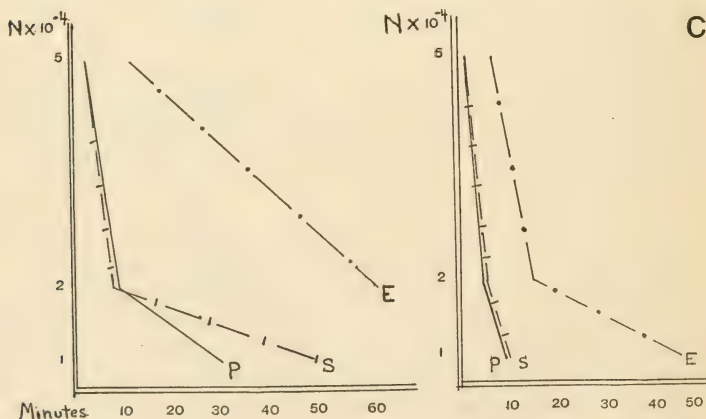
At whatever concentration they are tested, no matter what the acid, *Paramoecium* is much less resistant than *Euplotes*. Some specific difference is also observable between *Paramoecium*

and *Stylonichia*, for although their resistance may be identical at one concentration, it is often clearly different at another. The degree of difference in both cases often varies with dilution, as the following figures show. Each figure represents the quotient of the resistance of *Euplotes* by the resistance of *Paramoecium* or *Stylonichia*.

*Relative resistance*

	EUPLOTES / PARAMOECIUM			EUPLOTES / STYLONICHIA		
	0.0005 N	0.0002 N	0.0001 N	0.0005 N	0.0002 N	0.0001 N
HCl.....	4.6	3.3	5.0	4.6	3.0	5.0
Benzoic.....	1.6	4.5	3.4	1.5	5.0	2.4
Malonic.....	3.9	8.5		3.7	7.8	

Thus we have abundant evidence that each organism reacts in a characteristic fashion to dilution of the reagents. The figures also show that the degree of difference between the organisms is not the same in various acids of the same concentration; that is to say, each organism reacts in a characteristically different way to different acids.



Graph 2, C Relative resistance of *Paramoecium*, *Stylonichia*, and *Euplotes* to equinormal solutions of tartaric (left) and hydrochloric acids (right) at 20°C.

A further indication of specific differences is the fact that at any one concentration the relative toxic order of the series is different for each organism. Thus:

*Toxic order at 0.0002 N*

PARAMOECIUM	STYLONICHIA	EUPLOTES
Salicylic	Salicylic	Salicylic
Hydrochloric	Hydrochloric	Hydrochloric
Formic	Tartaric	Formic
Lactic	Lactic	Oxalic
{ Oxalic	{ Benzoic	Benzoic
{ Tartaric	{ Formic	Lactic
{ Malonic	{ Malonic	Citric
{ Benzoic	{ Oxalic	Phthalic
Acetic	Phthalic	Tartaric
Phthalic	Acetic	Malonic
Citric	Propionic	Acetic
Propionic	Citric	Propionic
Butyric	Butyric	Butyric
Valeric	Valeric	Valeric

With all three organisms the most toxic acids are salicylic and hydrochloric, while valeric and butyric are among the least toxic. But here the similarity ends. The order for *Stylonichia* disagrees with the order for *Paramoecium* in the position of tartaric, formic, malonic, and oxalic. The order for *Euplotes* is still more divergent, for citric and phthalic are more toxic, lactic is less so, and tartaric and malonic instead of being nearly as toxic as formic are here as little toxic as valeric. A few observations were made on some other forms which gave the following order of susceptibility: *Paramoecium aurelia* > *Paramoecium caudatum* > *Euplotes charon* > *Stylonichia pustulata* > *Urostyla grandis* > *Euplotes patella* > *Vorticella*. Additional evidence of specific differences is presented in the sections following.

Although several experiments upon the cilia of other organisms have been reported previously, it is almost impossible to compare them, for they were made by different observers under widely varying conditions. Thus in Barratt's experiments ('04,

'05) upon *Paramoecium aurelia*, the acids were not used consistently at the same concentrations, the temperature varied from 16° to 22°, and the method of washing injured the organisms. Weinland ('94) studied the oral epithelium of the frog at 18°, but made up his solutions at 0.001 M in physiological NaCl. Harvey ('14), in studying the cilia of the giant clam, used acids made up to 0.01 N in van't Hoff's solution and worked at a temperature of about 27°. We do not know in these cases whether the salts antagonize the acids (as was found by Loeb, '12, and by Osterhout, '14) or whether they reinforce its action (as found by Paul et al., '10), and consequently are not sure how far these experiments are comparable with other experiments in which the acids were made up in distilled water. Neither are Koltzoff's studies ('14) upon the cilia of *Carchesium* available for comparison, for, unlike the previously mentioned experiments, they deal with concentrations which do not kill the cilia, but only increase their viscosity.

There are, however, reports upon other material capable of showing specific differences clearly. The experiments of Landsteiner ('13) on acid agglutination of blood corpuscles and of Walbum and of Cunningham ('16) on haemolysis give clear evidence of specific differences in resistance. Ritter ('12) and Clark ('96) found striking differences in resistance among various mold spores, as did Heald ('96) with seedlings. Taylor ('17) found that the concentration of any acid necessary to clear a wound of bacteria varied with the organisms concerned and that the toxic order likewise varied. This is precisely the condition observed in the present experiments. Similar results have been obtained by Kopazcewski ('14) with enzymes. His observation that the optimal  $P_H$  was not the same for his usual preparation of maltose and for a well-dialyzed preparation indicates the importance of salts in determining acid resistance and may perhaps lead to an explanation of certain specific differences.



*C. Temperature*

In order to find out something of the nature of the toxic action, acids were tried at several temperatures between 10° and 30° as is indicated in table 1. The conditions are more readily seen in the curves shown below. In almost every case an increase above room temperature produces an increase in toxicity both to *Paramoecium* and to *Euplotes* and a decrease below room temperature generally produces a decrease in toxicity. If the length of life at, let us say, 10° is divided by the length of life at 20°, the figure so obtained (the temperature coefficient for 10°) can be used as a measure of the degree of influence exerted by these temperatures upon the toxicity of the acid. Table 2 gives the coefficients found for *Paramoecium* and *Euplotes*.

It will be observed that the coefficient for any one acid is not the same at all temperatures nor at all dilutions. For example, benzoic for the range 20° to 30° has coefficients of 2 at 0.0002 N and 3.88 at 0.0001 N, and for the range 10° to 20°, coefficients of 3 and 2.1, respectively. Often the more dilute solutions have higher coefficients than the more concentrated. It is also remarkable that the temperature coefficient for any given range is not the same for different acids even at equinormal concentrations. Thus at 0.0002 N the coefficients for the range 10° to 20° are salicylic 1.5, hydrochloric 2.2, benzoic 3, malonic 2.5, etc. All this would indicate that the mode of action of different acids is by no means the same and that the action of any one is markedly influenced by concentration and by temperature. Neither do the various acids affect *Paramoecium* and *Euplotes* in precisely the same way; if the coefficients for the two organisms be compared at corresponding temperatures and concentrations of the same acids, one finds that they are almost never the same.

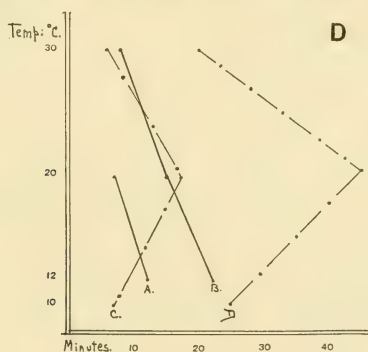
It is impossible at present to interpret these results fully, but some few conclusions suggest themselves. As is well known, a coefficient of from 2 to 3 for every increase of 10° generally indicates a chemical reaction, while a coefficient of less than 2 or over 4 is frequently associated with physical processes. Most

TABLE 2  
*Temperature coefficients*

	PARAMOECIUM				EUPLOTES		
	N/	12-20° <sup>1</sup>	15-25°	20-30°	12-20°	15-25°	20-30°
HCl.....	0.0005	4.3			1.7		
	0.0002	2.7		1.5	1.66		1.9
	0.0001	2.9	2.5	1.8	1.0	1.5	2.2
	0.00008			2.2			1.8
Oxalic.....	0.0005	3.3			1.1		1.2
	0.0002	1.9			1.0		
Tartaric.....	0.0005	2.8			1.2	1.3	2.0
	0.0002	2.2	2.3	2.57	1.0	2.0	2.0
	0.0001	1.4	2.0	4.28			
	0.00008			6.0			
Malonic.....	0.0005	2.4			1.38		1.62
	0.0002	3.2	2.77	1.0	1.0	1.17	
	0.0001	4.	3.5	1.7			
Formic.....	0.0005	1.8			2.4		
	0.0002	3.2	1.8	1.7	1.0	1.0	2.3
	0.0001	1.0	2.0	2.8			1.0
	0.00008			2.5			
Acetic.....	0.001	2.5		2.0	0.41		2.8
	0.0005	2.1			0.55		2.2
	0.0002	2.2					
Butyric.....	0.001	1.88			0.36		3.1
	0.0005	1.88			0.60		2.2
	0.0002	2.					
Benzoic.....	0.0005	2.8			1.6		
	0.0002	3.9	2.5	2.0	1.0	1.3	3.0
	0.0001	2.5	4.66	3.88			2.2
	0.00008			2.88			
Salicylic.....	0.0002	1.6			1.2		
	0.0001	2.1	1.6	2.5	1.0	1.0	2.0
	0.00008			3.84			

<sup>1</sup> Coefficients for 10° calculated from observations at 12° and 20°, except in the case of acetic and butyric acids, which were used at 10° and 20°.

of the coefficients for a change of  $10^{\circ}$  obtained with *Paramoecium* lie between 2 and 3, whether the temperature range is  $10^{\circ}$  to  $20^{\circ}$  or  $20^{\circ}$  to  $30^{\circ}$ , so chemical reactions would seem to be important. *Euplotes* also has coefficients between 2 and 3 for the range  $20^{\circ}$  to  $30^{\circ}$  which are to be explained in the same way. At lower temperatures, however, the condition for *Euplotes* is different. Over the range  $15^{\circ}$  to  $25^{\circ}$  and  $10^{\circ}$  to  $20^{\circ}$  the coefficients are generally little more than unity. A few coefficients of less than 2 were observed over this range for *Paramoecium*, but were decidedly the exception, instead of being the rule as with *Euplotes*. These low coefficients suggest complicating physical



Graph 3, D Effect of temperature upon the toxicity of hydrochloric and of butyric acids to *Euplotes*. A. HCl, 0.0005 N; B. HCl, 0.0002 N; C. Butyric, 0.001 N; D. Butyric, 0.0005 N.

factors perhaps masking a chemical reaction. Especially remarkable are the results obtained with *Euplotes* in acetic and butyric acids.

		LENGTH OF LIFE IN MINUTES				COEFFICIENTS	
		10°	15°	20°	30°	10-20°	20-30°
Acetic	0.001 N.....	7	10	17	6	0.41	2.8
	0.0005 N.....	25	20	45	20	0.55	2.2
Butyric	0.001 N.....	9	2	25	8	0.36	3.1
	0.0005 N.....	33	35	55	25	0.60	2.2

In the other acids (formic, tartaric, HCl, etc.) lowering the temperature either lessens the toxic action slightly or leaves it quite unaffected. Butyric and acetic, however, are more toxic at 15° than at 20° and still more toxic at 10°. Indeed, they are almost as toxic at 10° as at 30°. If they consistently become more toxic with falling temperature throughout the range 30° to 10°, this might be explained as due to rate of adsorption, since this is a process favored by low and diminished by high temperatures. But this cannot be the case, since at 20° they are less toxic than at 30°. Probably some further processes are involved, such as solubility, etc. A striking example of this sort has been described by Hans Meyer (quoted by Lillie, '16). The solubility of ethyl alcohol and chloral hydrate is increased by rise in temperature, while the solubility of salicylamid and monoacetin is decreased, and corresponding with this difference in solubility is the fact that the narcotic power of the former group is increased by rise in temperature, that of the latter decreased. Such conditions apparently complicate my results.

It appears from these experiments that temperature definitely influences the toxic action of acids. The degree of influence, however, varies with the acid, its concentration, the organism, and the temperature chosen. These irregularities show that the action is by no means simple and that it probably involves both physical and chemical factors.<sup>1</sup>

#### *D. Importance of the H ion*

The general order of toxicity obtaining in equinormal solutions gives some indication of the mode of action. Full data are given in table 1 and graph 1. The order runs: HCl > dibasic

<sup>1</sup> Lillie ('17) found that long exposure to sea-water at 2° to 6° would suffice to initiate development in starfish eggs, and that the rate of activation in butyric acid is greater at 6° than at 8° to 10°. These results are not strictly parallel with those obtained in the present experiments upon cilia, in which the lower temperatures (10° to 15°) increase the effectiveness of butyric acid, although alone they are not effective; but in both series of experiments Doctor Lillie's explanation seems applicable, viz., that a change in the physical condition of the structural colloids due to cold may alter their permeability or other properties and so affect their resistance to certain reagents.

and hydroxy acids > fatty acids except formic. This is roughly the same as the order of dissociation and indicates that the H ion is important. But if the H ion is the sole factor in determining toxicity, the order of toxicity and the order of dissociation should agree perfectly, which, as the following table shows, is not the case.

*Toxicity and dissociation*

TOXICITY AT 0.0002 N			DISSOCIATION CONSTANTS (SCUDDER)	
Paramoecium	Stylonichia	Euplotes		
Salicylic	Salicylic	Salicylic	HCl	
HCl	HCl	HCl	Salicylic	$1.4 \times 10^{-3}$
Formic	Tartaric	Formic	Oxalic	$6.0 \times 10^{-2}$
Lactic	Lactic	Citric	Lactic	$1.4 \times 10^{-4}$
{ Oxalic	{ Benzoic	Oxalic	Formic	$1.96 \times 10^{-4}$
{ Tartaric	{ Formic	Benzoic	Tartaric	
{ Malonic	{ Malonic	Lactic	Malonic	$1.64 \times 10^{-3}$
{ Benzoic	{ Oxalic	Phthalic	Benzoic	$6.64 \times 10^{-5}$
Acetic	Phthalic	Tartaric	Citric	$8.3 \times 10^{-4}$
Phthalic	Acetic	Malonic	Acetic	$1.85 \times 10^{-5}$
Citric	Citric	{ Acetic	Propionic	$1.42 \times 10^{-5}$
Butyric	Butyric	{ Propionic	Butyric	$1.71 \times 10^{-5}$
Valeric	Valeric	{ Valeric	Valeric	$1.77 \times 10^{-5}$

Still clearer evidence should be given by solutions of the various acids at the same H ion concentration, for if the H ion is the only toxic factor, they must all be equally toxic. Solutions were therefore made (without buffers) of all the acids in the series at four of the following concentrations, viz.,  $P_H$  3.5, 3.85, 4.0, 4.15, 4.75. The concentrations were determined by means of indicators tetrabromphenolsulphonphthalein and methyl red (Clark and Lubs, '17) in the usual way by comparison with acetic acid Na acetate standards. The method is not perfectly exact, but upon determining the  $P_H$  of a series of equinormal acids (0.0005, 0.0002, 0.0001 N) both colorimetrically and by calculation from the dissociation constants the indicators proved to be accurate to within about 0.1  $P_H$ .

The results obtained by this method are indicated in table 3 and graph 4. It will be seen at a glance that the toxicity of different acids of the same  $P_H$  is by no means identical. This



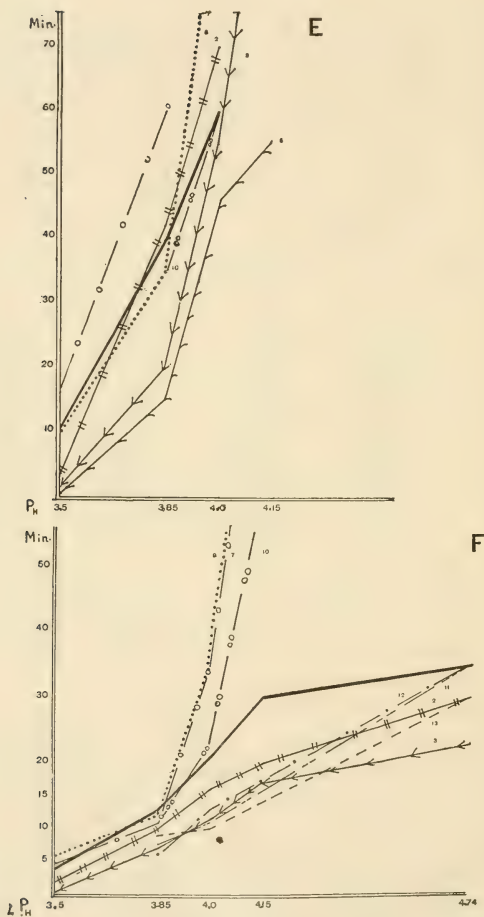


TABLE 3

*Toxicity of solutions of equal P<sub>H</sub> at 20°C. Time in minutes*

	P <sub>H</sub>	HCl	OXALIC	MALONIC	TARTARIC	CITRIC	FORMIC	ACETIC	PROPIONIC	BUTYRIC	VALERIC	BENZOIC	PHTHALIC	SALICYLIC
Paramoecium....	3.49	4	2 $\frac{1}{2}$	6	5	5	2	<2	<2	<1 $\frac{1}{2}$	<1			
	3.84	13	11	12	11	15	10	7	7	6	6	7 $\frac{1}{2}$	6	9
	3.99	21	35	35	23	23	16	11 $\frac{1}{2}$	10	11	11	13		10
	4.14	30	100	105	60	55	20	17	16	20	20			
	4.74	35					30	23	25	23		35	35	30
Stylonichia.....	3.49	4	5	3	3	3	2 $\frac{1}{2}$	<1	<1 $\frac{1}{2}$	<1 $\frac{1}{2}$	<1			
	3.84	15	20	23	13	20	10	6	7	6	6	8 $\frac{1}{2}$	6 $\frac{1}{2}$	17
	3.99	35	48	80	25	27	30	13	14	15	11	11	15	10
	4.14				75	75	50	30	28	30	23			
	4.74										30			
Euplotes.....	3.49	11	17	10	11	12	3 $\frac{1}{2}$	1 $\frac{1}{2}$	1 $\frac{1}{2}$	<1	<1			
	3.84	40	60	35	35	40	42	20	19	15	15	11	10	35
	3.99	60		100	60	60	70	55	55	45	33	33	40	30
	4.14							100	97	55	55			
Vorticella.....	3.49	15	15	15	15	20	5	<3	<2	<1	<1			
	3.84	55	75	90	90	90	55	15	15	25	25	16	16	20
	3.99	70					80	35	40	75		33	40	30

confirms the idea suggested above that the H ion is not the only toxic agent in acids. It is interesting to compare the order of efficiency found with equinormal and equal P<sub>H</sub> solutions. The general conditions can be made out from curves 1 and 3; stated in tabular form, they are as set forth on page 460.

The general order for Paramoecium in equinormal solution is hydrochloric > salicylic > formic > dibasic and hydroxy acids > acetic and butyric; but in solutions of equal P<sub>H</sub> acetic and butyric become equal in toxicity to the cyclic acids, and the dibasic and hydroxy acids together with hydrochloric become least toxic of all. Formic, which in equinormal solution is more toxic than the other fatty acids, probably because of its greater dissociation, becomes less toxic than the rest when the solutions have the same P<sub>H</sub>. Somewhat the same conditions hold with Euplotes. In solutions of equal P<sub>H</sub> salicylic retains

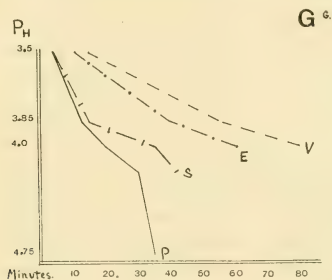
*Order of toxicity*

PARAMOECEUM		PARAMOECEUM	
0.0005 N	0.0001 N	P <sub>H</sub> 3.5	4.0
Salicylic Hydrochloric Formic { Tartaric { Benzoic { Oxalic Malonic Acetic Butyric	Salicylic Hydrochloric Tartaric { Benzoic { Malonic Formic Oxalic { Acetic { Butyric	{ Valeric Butyric Propionic Acetic Formic Oxalic Hydrochloric { Tartaric { Malonic	{ Propionic Salicylic Benzoic Acetic Butyric Valeric Formic Hydrochloric Tartaric { Malonic Oxalic
EUPLOTES		EUPLOTES	
0.0005 N	0.0002 N	P <sub>H</sub> 3.5	4.0
Salicylic { Formic { Benzoic HCl Oxalic Tartaric Malonic Acetic Butyric	Salicylic HCl Formic Oxalic Benzoic Tartaric Malonic Acetic Butyric	{ Valeric Butyric Propionic Acetic Formic Malonic { Tartaric { HCl Citric Oxalic	Salicylic { Valeric Benzoic Butyric Propionic Acetic HCl { Tartaric { Citric Formic Malonic

its position at the top of the list, the other cyclic and the fatty acids (except formic) stand next, while the hydroxy acids and hydrochloric are much less toxic than the rest. Evidence of like nature has been obtained in experiments upon very different sorts of material. In every case the relative efficiency of equinormal solutions failed to parallel their H ion concentration exactly, and solutions of equal P<sub>H</sub> were always unequal in effect.

Only one conclusion is possible. The H ion is an important factor in the physiological effects of acids, but at least in organic acids some other factor or factors are involved which makes them more effective than their P<sub>H</sub> alone would lead one to expect.

These experiments with solutions of equal  $P_H$  also give evidence of specific differences in resistance. The order of resistance to every acid is *Paramoecium* > *Stylonichia* > *Euplotes* > *Vorticella*. There is considerable evidence of specific difference in resistance to  $H^+$  among other organisms, such as bacteria (Michealis, '14, '15; Kemper, '16) it is noteworthy, however, that here the limit of tolerance of a particular species is not the same in all acids (Wyeth, '18). This is true also of *Paramoecium*; thus, a concentration of  $P_H$  3.5 kills *Paramoecium* in four minutes in HCl, five minutes in tartaric, less than one minute in valeric, etc., and so with the other organisms. The order of



Graph 5, C Relative resistance of *Paramoecium*, *Stylonichia*, *Euplotes*, and *Vorticella* to equal  $P_H$  of hydrochloric acid. Temperature 20°C.; time in minutes.

toxicity of the different acids at the same  $P_H$  also is slightly different for each organism (table 3) as is the case with equinormal solutions. It will be remembered that Kopacweski ('14) found the same condition in enzymes.

#### *E. Importance of anion and molecule*

Although there is ample proof from many sources that the effect of an acid is not determined solely by its  $H$  ion concentration, there is very little evidence indicating exactly the part played by anion and molecule. One way of approaching the matter is to compare the relative toxicity of a series of acids

with the relative toxicity of their salts. I have not experimented with salts, but there are interesting data to be had from other experiments. The order of toxicity to *Lupinus* seedlings (Kahlenberg and True and True, '96) is as follows:

Acids equinormal, salts equimolecular.

Acids:  $\text{HCl} > \text{benzoic} > \text{salicylic} > \text{formic} = \text{propionic} > \text{butyric}$ .

Salts:  $\text{Na—benzoate} > \text{salicylate} > \text{formate} > \text{propionate} > \text{butyrate} > \text{chloride}$ .

The order for equinormal acids is almost the same as that obtained in my experiments: that is,  $\text{HCl}$  is more toxic than benzoic, and benzoic in turn is more toxic than the fatty acids. Since there is little difference in dissociation among the salts, their order, unlike that of the acids, is a measure of the relative toxicity of the anions. Salts of the cyclic and of the fatty acids are more toxic than  $\text{NaCl}$  and must therefore have more toxic anions. These are salts of the very acids which in solutions of equal ionic concentration proved to be most toxic to cilia. Practically the same conditions hold in the haemolysis of blood corpuscles (Fühner and Neubauer, '07, and Hoeber, '10).

Acids:  $\text{HCl} > \text{formic} > \text{acetic} > \text{propionic}$ .

Salts:  $\text{Na—Salicylate} > \text{benzoate} > \text{formate} > \text{acetate} > \text{butyrate}$ .

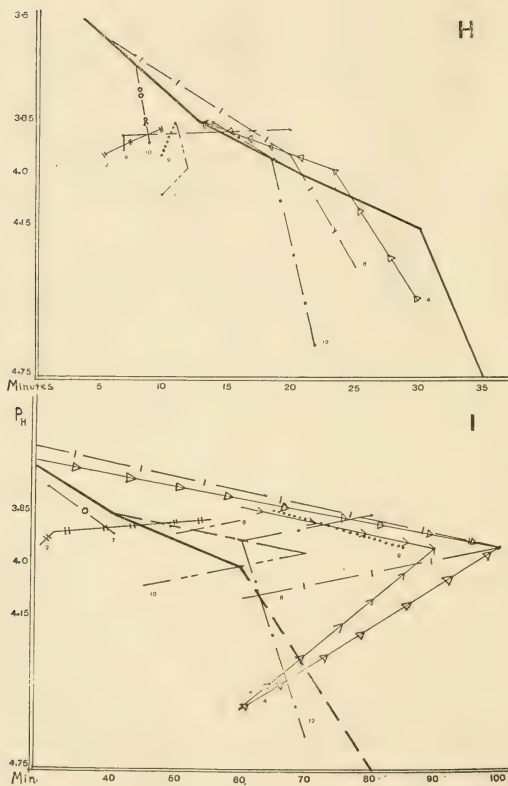
The similarity between these experiments with equimolar salts and my experiments with acids in solutions of equal  $P_H$  makes it seem probable that the acids which are most toxic in these solutions owe their effectiveness to the anions as well as to the  $\text{H}$  ion. The fact that a different order of toxicity obtains in normal solutions may perhaps be explained as follows: If an acid is not highly dissociated, even though it has a slightly toxic anion, it is less toxic than a more highly dissociated acid with a non-toxic anion (for instance, butyric as compared with  $\text{HCl}$ ). If, however, the anion is very toxic, as is the case with salicylic, the acid in spite of its slighter ionization may equal or even exceed a more completely ionized acid such as  $\text{HCl}$  in toxicity.

Another method of investigating this point has been suggested by Klocman ('14). To various concentrations of a weak acid a



fixed amount of HCl is added; this depresses the dissociation of the weaker acid and produces a solution containing chiefly the organic acid molecule,  $H^+$  and  $Cl^-$ . If the anion of the organic acid is toxic, this mixture should be less toxic than the original solution, provided the H ion concentration is not greatly altered. Kloeman found that the acetic anion was toxic, but did not try the method with other acids. In the present experiments the method was somewhat modified and was applied to a number of organic acids of the same concentration (0.0002 N). The  $P_H$  of each solution was first determined by the use of indicators, then enough HCl was added to increase the  $P_H$  slightly, by a known amount when the toxicity of each of these mixtures had been determined HCl was again added, increasing the  $P_H$  decidedly. With each addition the ionization of the organic acid was depressed further, until finally most of it was in molecular form and the H ions were derived chiefly from the HCl. By determining the toxicity of the acids alone and again after each addition of HCl it was possible to follow the effect of the decreasing dissociation of the acids and at every stage to compare them with HCl of the same H ion concentration. The figures obtained in this experiment are recorded in table 4 and expressed graphically in graph 4.

It will be seen at once that some of the acids become less toxic as the H ion concentration is increased, formic lactic and acetic, for example, in the experiments with *Paramoecium*. This means that as the anions decrease in number the acid becomes less harmful. In other cases, addition of HCl increases the toxicity, but only until it is equal to that of HCl of the same  $P_H$ . Thus phthalic by itself is more toxic than HCl, but as ionization is depressed approaches it more closely; that is, in graphic form, the curves are at first distinct, but as  $P_H$  is increased through the addition of HCl the phthalic approaches the HCl curve and finally coincides with it. The same is true of oxalic, benzoic, citric, acetic, propionic, butyric, valeric. In these cases, too, the anion must be toxic, since depression of ionization does away with the greater toxicity of the organic acid as compared with HCl of the same  $P_H$ . Since these acids



Graph 6, H, I Effect upon toxicity at 20°C. of depressing ionization. (Paramecium above and Euplotes below.)

when in molecular form are not more toxic than HCl of the same  $P_H$ , it seems probable that their molecules do not exert a toxic action.

The curves for Euplotes present a slightly different picture. Certain acids (oxalic, malonic, tartaric) become more toxic as ionization is depressed. It will be noted that these are all dibasic acids. They are, however, so nearly like HCl that it is difficult to draw any definite conclusions from the results. With

TABLE 4  
*Effect of depressing ionization*

ACID	$P_H$			LENGTH OF LIFE IN MINUTES					
	Alone	Plus HCl		Paramoecium			Euplotes		
		II	III	I	II	III	I	II	III
Formic.....	3.95	3.9	3.87	5½	7½	10	25	27	55
Acetic.....	4.3	3.97	3.85	22	23	14	60	90	60
Propionic.....	4.7	3.97	3.6	30	21	6	60	100	12
Butyric.....	4.45	3.97	3.85	30	23	13	70	110	
Valeric.....	4.6	3.97	3.85	45	25	13	70	105	60
Salicylic.....	3.6		3.49	2		1½	2½		1½
Benzoic.....	4.05	3.97	3.85	11	12	11	45	70	40
Phthalic.....	4.6	3.95	3.85	22	18	14	70	60	80
Lactic.....	3.9	3.87	3.87	7	7	20	50		60
Oxalic.....	3.9		3.6	9		8	40		15
Malonic.....	3.95		3.85	10		11	85		65
Tartaric.....	3.95	3.9	3.87	9	9	9	60		45
Citric.....	4.1	3.95	3.6	25	20	6	60	100	25

the first addition of HCl the toxicity of many of the other acids is decreased (lactic, formic, acetic, propionic, butyric, valeric, citric, benzoic), proving that their anions must be toxic. Indeed the decrease proceeds so far that the mixtures of these acids with HCl become much less toxic than HCl of the same  $P_H$  alone. Phthalic does something of the same sort, viz., first becomes equal to HCl and then falls far below it in toxicity. When the second lot of HCl is added to some of the acids (benzoic, citric, acetic-valeric) a curious result is obtained. The toxicity, instead of being decreased as by the first addition, is

sharply increased, until the mixture again becomes equal to pure HCl. This is indicated though much less clearly in the Paramoecium curves for citric and acetic, propionic, etc., but not for benzoic. Until further work is done I cannot with assurance offer a definite explanation of this condition, but the results so far suggest that the molecule is to some extent capable of antagonizing the action of the H ion. Thus as dissociation is depressed and the anion is removed from the sphere of action, antagonism between molecule and H ion may cause the mixture to become less toxic than HCl of the same  $P_H$ , and this antagonism may cease to be important only when the H ions largely out-number the molecules present.

From this series of experiments we find that the following anions are toxic. To Paramoecium: formic, acetic, propionic, butyric, valeric, benzoic, phthalic, lactic, oxalic, malonic, tartaric, citric. To Euplotes, all of these except oxalic, tartaric, lactic, and possibly malonic. These findings agree with the conclusions suggested by the previous experiments, viz., that the anions as well as the H ions are sometimes toxic, and that the same acid need not act upon different organisms in exactly the same way.

#### *F. Nature of the toxic action*

At higher concentrations (0.0005 N) Paramoecium discharges trichocysts, and as it coagulates turns rapidly opaque without great swelling, but at 0.0002 N the swelling is pronounced and no trichocysts are discharged. At death the protoplasm becomes granular and the nucleus stands out sharply. Vorticella and Euplotes, too, become granular and in all the vacuoles grow large and rigid before death. The cilia themselves swell and become sticky, the beat grows irregular and slows until finally the cilia dissolve (see also Koltzoff on Carchesium). Stylonichia swells and some of its vacuoles increase in size, then anteriorly, at a point near the edge, the protoplasm dissolves and releases large apparently insoluble droplets. The cilia stop only when the body is completely disintegrated and must therefore be more resistant than the rest of the cell. Careful obser-

variations under high power, such as Greeley made, of the changes induced in the physical state of the protoplasm might throw light upon the nature of the effect of various acids upon the tissue of different organisms.

It is certain that the H ion is exceedingly important in the swelling of muscle (Loeb, '98, no swelling in hypotonic salt solution unless acid is also present) as well as in the swelling of other hydrophilous colloids, such as fibrin, gelatin, etc. (Loeb, '19; Proctor, '16; Fischer, '18). It seems probable that the H ion is also important in the swelling of cilia, perhaps the specific differences observed are due in part to differences in the colloids present.

Another factor seems to be surface tension. Working with salts, Clowes ('16) found that slight changes in surface tension produce great changes in the physical state of oil-soap emulsions, and he suggested that the physiological effects of these salts were due to changes induced in the protoplasmic emulsion. There is evidence in other experiments of profound changes in the surface tension of protoplasm induced by acids. Hamburger ('13) found that ingestion of India ink or charcoal particles by phagocytes was depressed in too high concentrations of fatty acids, but was stimulated in more dilute solutions, and this stimulating action he attributed to changes in surface tension. Koltzoff ('14) used *Carchesium* (a colonial relative of *Vorticella*) for the same purpose, and found that at some concentrations acids increased the ingestion of ink particles, and at slightly higher concentrations produced visible evidence of change in surface tension in the accumulation of ink particles on the cilia. The optimum concentration for this softening effect as well as for stimulation of phagocytosis varied with the acid used.

Another factor in toxicity is lipid solubility. Benzoic acid is much more lipid-soluble than the fatty acids and should therefore penetrate a lipid rich membrane and attack the cell contents more rapidly. The fact that benzoic is more toxic than valeric to *Euplotes* but not to *Paramoecium* suggests the presence of some lipid in *Euplotes* which is not present in the same



concentration in *Paramoecium*. In many experiments upon other material benzoic has been found very effective, and similar specific differences in susceptibility have been observed which suggest differences in lipoid content (Harvey, '14; Crozier, '16; Haas, '16; Loeb, '13).

Peters and Burres ('09) conclude from their experiments upon the toxicity of Cu salts to *P. aurelia* that toxic effect is due to injury to an essential enzyme and not to direct chemical injury of the protoplasm. If the normal metabolic processes of the cell are interrupted, as they would be by the failure of an important enzyme, it is obvious that the chemical and physical balance of the whole cell would be affected. Possibly something of the sort may be involved in the toxic action of acids upon the cilia of *Paramoecium*, *Euplotes*, etc., and may account for the specific differences involved. This explanation is of course purely hypothetical at present. There are many other possible factors in toxicity, but conclusions are difficult and uncertain the moment one ventures beyond very simple and obvious comparisons.

#### SUMMARY

It is found that the relative toxicity of a series of acids varies decidedly with the concentration, and therefore it is unwise to base conclusions as to mode of action upon results obtained with only one concentration. The fact that power of penetration also varies greatly with concentration makes it probable that the same is true of many of the physiological effects of acids. There are also great differences in the effects of acids upon different species: even organisms so closely related as the infusoria used in these experiments show characteristic differences. When tested in the same solution, one species may be two, four, or even twenty times as resistant as another; and in addition the order of toxicity of the series of acids is somewhat different for each species. Another factor in determining relative toxicity is temperature. Ordinarily toxicity increases with increase in temperature and decreases with decrease in temperature, but the degrees of influence exerted by temperature

varies with several factors—the species used, the acid, and the concentration. The acids are unequally affected by different temperatures. The coefficients for *Paramoecium* and *Euplotes* are scarcely alike at any point. Most of the coefficient of  $10^\circ$  for *Paramoecium* lie between two and three and suggest a chemical reaction. The coefficients for *Euplotes*, however, are rarely so high and agree better with the idea of a physical factor. Indeed, with butyric and acetic, though not with any of the other acids tried, a decrease in temperature below  $20^\circ$  accelerates the toxic action upon *Euplotes*, so that their toxicity at  $10^\circ$  is nearly as great as at  $30^\circ$  and much greater than at  $20^\circ$ .

A comparison of the acids at various equinormal concentrations shows that a rough parallelism exists between toxicity and degree of dissociation, as would be expected if the H ion is the most important factor in toxicity. When, however, the acids are compared in solutions of equal  $P_H$ , it is evident that other factors must enter, for the toxicity of the various acids is markedly different. The order of toxicity, unlike that of equinormal acids, is closely similar to the toxic order of salts of these acids. This suggests that the anions of at least some must be toxic. Additional evidence of the toxic action of certain anions (mainly of the fatty and the cyclic acids) is afforded by the action of acid mixtures, in which the ionization of the organic acid is progressively diminished by addition of a strong acid, HCl.

All the acids used bring about swelling followed by precipitation of part of the cell contents and by solution of the cilia. There is clear evidence of change in surface tension, for the cilia always become sticky before they stop beating. It seems probable that the toxic effect of the various acids depends upon their solubility in the tissue as well as upon capillary activity and the changes in colloidal state wrought by the H ion.

## CONCLUSIONS

1. The order of toxicity of a series of acids varies with the concentration, the temperature, and the species. The action is therefore not simple.

2. The H ion is an important factor, for the toxic order of equinormal solutions is roughly parallel with the order of dissociation.

3. The H ion is not the only factor in toxicity, for in solutions of equal  $P_H$  the acids are not equal in toxicity.

4. Depression of ionization shows that the anions of certain acids are toxic to both *Paramoecium* and *Euplotes*, viz., formic, acetic, propionic, butyric, valeric, citric, benzoic, phthalic, salicylic. The anions of oxalic, tartaric, lactic, and malonic are toxic to *Paramoecium*, but not to *Euplotes*.

5. The temperature coefficients indicate that both chemical and physical reactions are probably concerned in the toxic effect of acids.

6. A most marked irregularity is shown by acetic and butyric acids, in that their toxicity to *Euplotes* (though not to *Paramoecium*) is greatly increased by temperatures below as well as above 20°C.

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Resumen por el autor, J. A. Dawson.  
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### Estudio experimental de un *Oxytricha* amiconucleado.

#### I. Estudio del animal normal con una descripción de su canibalismo.

Durante 289 generaciones se ha cultivado *Oxytricha hymenostoma* Stokes—desde el 10 de Julio de 1917 hasta el 17 de Noviembre de 1917— y el autor conserva aun vivos cultivos en masa de los descendientes de estos cultivos. No ha visto un micronúcleo en ningún estado de los diversos animales, durante la historia de los cultivos. No ha habido singamia, si i bien el autor cree que el organismo en cuestión ha pasado frecuentemente por un estado físico semejante al en que tiene lugar la singamia. En este momento: a) los animales se fusionan por parejas a semejanza de la unión de los conjugantes, y, b) tiene lugar el canibalismo. Cuando acaece la fusión en parejas, los animales o bien permanecen fusionados hasta su muerte o se separan. En caso de separación los organismos en cuestión continúan reproduciéndose y no presentan síntoma alguno de estar en una condición de depresión. Cuando el canibalismo tiene lugar los animales ingeridos son digeridos rápidamente y los que sobreviven vuelven a adquirir el tamaño y estructura típicos. El canibalismo produce como efecto el aumentar ligeramente la cantidad de divisiones durante un corto tiempo. No se ha observado enquistamiento. Esta raza amiconucleada de *Oxytricha* puede vivir indefinidamente, en apariencia, bajo condiciones ambientes favorables sin conjugación, autogamia o endomixis.

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## AN EXPERIMENTAL STUDY OF AN AMICRONUCLEATE OXYTRICHA

### I. STUDY OF THE NORMAL ANIMAL, WITH AN ACCOUNT OF CANNIBALISM

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TWENTY FIGURES

#### CONTENTS

1. Introduction.....	473
2. Material and methods.....	475
3. The normal animal.....	477
1. History of culture A.....	479
2. Attempts to obtain conjugation.....	482
3. Cytology of <i>Oxytricha hymenostoma</i> .....	483
4. Cannibalism.....	485
1. Observations on the process of swallowing.....	486
2. Digestion of the ingested animals.....	490
3. The effect of the culture medium.....	494
4. The physiological effect of cannibalism.....	498
5. Discussion.....	503
6. Summary.....	506

#### 1. INTRODUCTION

The presence of a macronucleus and a micronucleus has long been recognized as one of the striking features which aids in marking off the Infusoria from the rest of the Protozoa, and there is abundant evidence tending to prove that the micronucleus is an indispensable part of the organism during both conjugation and endomixis. In all the ciliates where the micronucleus has seemed to be absent in the vegetative stages of the life history, a careful study has invariably revealed its presence at the onset of sexual phases.

Thus, Neresheimer ('07) and Metcalf ('09) found in *Opalina*, a parasitic ciliate which has but one kind of nucleus, that a certain type of syngamy took place by the combination of uninucleate gametes, the nuclei of which contained chromatin comparable to that of a micronucleus. In *Trachelocerca phenicopterus*, a free-living holotrichous ciliate, micronucleus and macronucleus are merged completely in the vegetative state, but the micronucleus appears during conjugation (Lebedew, '08). *Ichthyophthirius multifiliis*, a parasitic holotrichous infusorian has a separate macronucleus and micronucleus in certain stages, but in others the micronucleus is contained within the former (Neresheimer, '08, and Buschkiel, '11). Calkins ('12) found in *Blepharisma undulans*, a free-living heterotrichous ciliate, that the micronucleus is contained within the macronuclear membrane in the ordinary vegetative stages, but becomes separated from the macronucleus during conjugation.

In the course of experiments on certain hypotrichous ciliates the writer obtained a species of *Oxytricha* in which, upon staining, no definitive micronucleus was visible. Recalling the work of previous investigators on apparent amiconucleate ciliates where a micronucleus manifested itself during conjugation, it was decided to breed the organism and attempt to obtain conjugation. In addition, a culture was carried by the daily isolation method to ascertain the ability of this species to live indefinitely, reproducing by division without conjugation. Although careful cytological studies have been made throughout the entire course of the experiments, no definitive micronucleus has at any time been observed, but certain interesting and suggestive results have been obtained. Under conditions favorable to conjugation, the reactions of this organism closely resembled those of a conjugant, but instead of completing the normal process of fusion, it was found that the animal invariably became cannibalistic, and that there was a strong tendency for pairs to fuse dorsally, thus forming a double animal or 'twin.'

An account of the investigation of these phenomena will be presented as follows:

PART I. 1. Study of the normal animal with attempts to induce conjugation. 2. Cannibalism.

PART II. The formation of double animals or 'twins.'

I take pleasure in acknowledging my great indebtedness to Prof. Lorande L. Woodruff, at whose suggestion this investigation was begun, for his advice and criticism throughout the entire course of this study. I also wish to express my thanks to Prof. Alexander Petrunkevitch for assistance in making the microphotographs and to Dr. Rhoda Erdmann for valuable suggestions in technique.

## 2. MATERIAL AND METHODS

The hypotrich used for these experiments was obtained originally in the fall of 1915 in some débris, from a pond in the vicinity of New Haven, which had been placed in a laboratory aquarium. A pedigreed culture was carried from February 22 to June 21, 1916, when it was discontinued. Since this time stock from the same culture has been in the laboratory aquaria, and an individual of this species which was isolated from an aquarium in the Osborn Zoological Laboratory on July 10, 1917, has supplied all the animals used in this experimental study.

The species has been found to agree very closely with the description given by Stokes ('88) for *Oxytricha hymenostoma*. Systematic works do not usually describe specifically the macronucleus or micronucleus, more stress being laid on the external structures for classification. As the absence of a micronucleus and the slight differences shown by this form from *Oxytricha hymenostoma* Stokes were not assumed to be of specific value, no attempt is at present made to establish a new species, the hypotrich being identified provisionally as *Oxytricha hymenostoma*.

On July 11th the animal, which was isolated on the previous day, had divided twice, and the four resulting organisms were isolated on depression slides in about five drops of culture medium to form the four lines of culture A. In this culture and in all



subcultures carried during the work the animals have been isolated daily by means of a capillary pipette and a Zeiss binocular, oculars 2 and objectives F55. Separate pipettes have been used for each culture, and these have been carefully sterilized before using by thorough boiling or by heating in a small gas flame. 'Stock' cultures of the animals remaining in each line after the daily isolation have been kept on the depression slides for a day or two, so that an animal could be replaced in case of accident. Both line and stock cultures have been kept in moist chambers. For special purposes animals have been allowed to multiply on these stock slides, thus forming small mass cultures. Small Petri dishes (35 cc. capacity) have been used for growing mass cultures, and this method has been found to have several advantages, since a Petri dish of this size may be quickly and accurately examined with the binocular microscope, while the cover, although fitting loosely, prevents contamination and permits but very slow evaporation.

The culture medium used was varied as much as possible. The basis of the medium was hay boiled thoroughly in tap-water. To this was added from time to time various decaying vegetable and animal material from laboratory aquaria and ponds. Occasionally living snails were finely chopped and added to the medium. In all cases contamination was prevented by thorough boiling—the medium being usually allowed to stand overnight before using. Thus an attempt was made to supply the animals with as varied an environment as possible, since such a 'varied environment' (Woodruff, '08, '11) has been used for the continued maintenance of *Paramecium aurelia* in pedigreed cultures.

Attempts to obtain conjugation were made, following in general the methods used by Maupas ('89), Calkins and Cull ('07), Jennings ('10), and Woodruff ('14), and others. Stock cultures were also frequently allowed to multiply on depression slides and in small Petri dishes for the same purpose. In all cases the result was the same, i.e., invariably animals were obtained which made what have been interpreted as abortive attempts to conjugate.

Specimens for cytological study were fixed in corrosive sublimate (saturated solution) with 1 per cent acetic acid and in Schaudinn's sublimate-alcohol (strong). Various stains were tried, including Heidenhain's iron hematoxylin, Delafield's haematoxylin, borax carmine and picro-carmine. Differentiation was made with acidulated alcohol (70 per cent alcohol and 0.01 per cent HCl). In general Delafield's hematoxylin has been found to give the best results. Eosin and tetra-brom-fluoresceic acid were used as counterstains, the latter being especially good for staining external structures as cilia, cirri, and membranelles.

Single animals were fixed and stained on depression slides. The animal was kept under observation during the whole process except for the period necessary for staining, when the slide containing the specimen was placed in a moist chamber. The various fluids used in the process were drawn off with a capillary pipette or filter-paper, the only transfers being when the animal was isolated for fixation and when the same animal, now ready for mounting, was placed on a thin glass slide. Specimens were cleared and mounted in cedar oil or in xylol and damar. Total mounts thus made were prevented from being crushed by placing pieces of hair of suitable lengths under the cover-slips. At various times smears of large numbers of animals were made for general study. Sections,  $4\mu$  in thickness, have been prepared, but it has been found that study of these has added nothing to the cytological details which on account of the thinness of the animal may be studied thoroughly in total mounts.

### 3. STUDY OF THE NORMAL ANIMAL

Although hypotrichous forms have been carefully investigated, no worker has as yet been able to breed these ciliates indefinitely by the daily isolation method without conjugation. Maupas ('88) carried cultures of *Stylonychia pustulata* without conjugation for 316 generations; *Stylonychia mytilus*, similarly, for 319 generations; *Onychodromus grandis* and *Oxytricha* for 320 to 330 generations. He concluded that in all these cases death was due to old age, no opportunity having been allowed for 'rejuvenation' by conjugation. Joukowsky ('98) kept one of four

cultures of *Pleurotricha lanceolata* for 458 generations without conjugation or degeneration. Woodruff ('05), working with *Oxytricha*, *Pleurotricha*, and *Gastrostyla*, found that the number of generations in the life cycles of these species was not at all constant. The culture of *Oxytricha* was longest lived (860 generations) and its life was prolonged by artificial stimulation. The same author ('13), referring to his early culture of *Oxytricha*, stated that he believed "that if an entirely suitable environment had been secured this culture would have given evidence of unlimited power of reproduction by division without conjugation as my present *P. aurelia* has done." Popoff ('07) kept a culture of *Stylonychia mytilus* for three and a half months, at the end of which time it died during a deep 'depression period.' He concluded that the cause of depression was not accidental changes in environment, but lay in the organism itself. Enriques ('03, '05, etc.), working with *Oxytricha* and *Stylonychia*, concluded that the cause of depression and subsequent death was prolonged poisoning with bacterial poisons, and that "agamic reproduction can be continued as long as one likes if the technique is good and bacteria are not too numerous. No change of food is necessary." Baitzell ('12) kept *Stylonychia pustulata* for 572 generations. He did not observe any "appearance of abnormal or degenerating animals," but the fission rate of the cultures gradually declined until death occurred. This he interpreted as due to unsuitable cultural conditions. In later work ('14) he succeeded in keeping a culture of *Pleurotricha* in a beef-extract medium for 943 generations. He also carried mass cultures of the same organism in test-tubes and without conjugation for twenty-two months. From this he concluded that *Pleurotricha* would "apparently live indefinitely without conjugation or artificial stimulation."

The evidence from these investigators proves that, if a life 'cycle' exists in hypotrichs, it is subject to great variation, depending largely, if not entirely, upon the environment. Artificial stimulation has been shown to have the effect of prolonging the life 'cycle,' and the most recent work indicates that this 'cycle' may be prolonged indefinitely if the organism is provided

with suitable environmental conditions, i.e., the 'cycle' is merely an artifact resulting from unfavorable conditions.

### 1. *History of culture A*

This culture was begun on July 11, 1917, from the four descendants of the *Oxytricha* isolated on the previous day. The culture was carried by daily isolations until November 17, 1917, when division stopped in all the four lines and the animals, after living one to two weeks without fission, finally died. An attempt was made to continue the race by isolating individuals from the stock cultures of this date, but this proved unsuccessful. However, animals taken from stock cultures on November 2nd have been kept living in small mass cultures (without conjugation) and are still reproducing (April 12, 1918). This fact, reviewed in the light of the work of Baitsell ('14) on *Pleurotricha*, seems to indicate that if suitable cultural conditions are given, there is a strong possibility of unlimited asexual reproduction.

The curve (fig. 1) showing the average division rate of culture A and hence the general physiological condition of the animals during the life of the culture has been plotted for five-day periods. This was done, according to the usual method, by averaging the divisions of the four lines together and then averaging the result for five-day periods. A study of this curve shows that the division rate during the first month was much higher than at any other time during the course of the experiments. The highest point was reached between July 31st and August 5th, with an average rate of 4.65 divisions per day. From this time on there were fluctuations<sup>1</sup> in the division rate which showed a

<sup>1</sup> A 'rhythm' was originally defined by Woodruff ('05) as "a minor periodic rise and fall of the fission rate, from which recovery is autonomous." More recently Woodruff and Erdmann ('14) have shown that there is a causal relation between endomixis and rhythms in *Paramecium*. Since endomixis has not been demonstrated in hypotrichs and, further, since it apparently does not occur in this form, it has been deemed inadvisable to apply the term 'rhythms' to the fluctuations<sup>1</sup> in the division rate of *Oxytricha hymenostoma*. It should be noted that Fermor ('13) described an internal nuclear reorganization process in *Stylonychia* during encystment. Here the macronuclei degenerated; the micronuclei fused and later by division produced new micronuclei and

steadily downward trend until the period September 9th to Sept. 14th, which had an average of 1.3 divisions per day. From this time on there occurred twice a general rise and fall in the division rate, and at the conclusion of the second drop in the rate the four lines of culture A died out.<sup>2</sup>

Although it has not been found possible to continue the race by the daily isolation method, it has been kept alive to date (April 12th) without conjugation and, to all appearance, in a good physiological condition in the Petri-dish cultures. The method of carrying these cultures was as follows. Several animals selected from stock slides were placed in a small Petri-dish in about 20 cc. of the same kind of medium used for culture A. This culture was examined carefully each day to preclude the possibility of unobserved conjugation. When the organisms had multiplied for a few days, isolation of several individuals was made to a fresh culture of the same kind, and the process has been repeated to date. On account of the ease with which accurate observations on these cultures may be made, it is believed that no ex-conjugants (assuming conjugation is possible in this species) have been isolated for the continuance of the race.

The result of this experiment on *Oxytricha hymenostoma* is very similar to that obtained by Baitsell ('14) with *Pleurotricha*, the life cycle of which, he found, could be prolonged indefinitely by use of a hay-infusion medium in test-tube cultures. It thus

macronuclei. Fermor states that encystment took place in apparently quite normal cultures and regards the process just described as a substitute for conjugation, since the latter process did not occur in the cultures of *Stylonychia*. Thus the possibility is indicated that in hypotrichous forms during encystment a process occurs which is, in a way, analogous to endomixis (Woodruff and Erdmann, '14) in *Paramecium*. It should be emphasized, however, that the process described by Fermor involves a fusion of micronuclei and therefore is autogamy rather than endomixis as defined by Woodruff and Erdmann in their *paramecium* work, since they found no micronuclear fusion. In the organism studied in this work I have observed no encystment, and it is difficult to conceive how a similar nuclear reorganization process might occur in *Oxytricha hymenostoma*, since no definitive micronucleus is present.

<sup>2</sup> A culture of *Oxytricha* sp. carried in the same medium and under absolutely the same conditions reached 400 generations, outliving the culture (A) by two and a half months, thus showing that the medium was apparently more suitable for other species of hypotrichs.



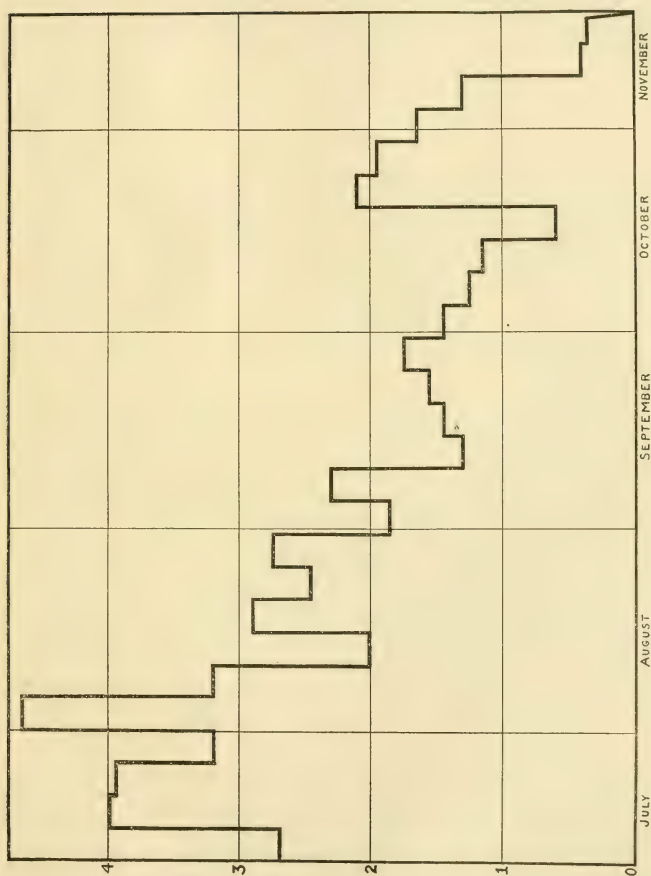


Fig. 1 Complete history of *Oxytricha hymenostoma*, Culture A, from July 11, 1917, to November 17, 1917. Rate of division averaged for five-day periods. The ordinates (continuous line) represent the average daily rate of division of the four lines of the culture.

is apparent that environmental conditions are responsible for many of the so-called life 'cycles' of certain infusorians, and that there is no reason to believe that, given suitable cultural conditions, this 'cycle' may not be indefinitely prolonged, or, in other words, that a 'cycle' exists at all.

## *2. Attempts to obtain conjugation*

As is well known, in the nuclear phenomena during syngamy in all hypotrichida as yet studied, the micronucleus plays the chief part—the macronucleus invariably degenerating and being formed anew from the micronuclei resulting from the division of the synkaryon. It therefore naturally was concluded that a study of this process in *Oxytricha hymenostoma* would be the crucial test in the attempt to determine the presence or absence of a micronucleus.

It was soon found that stock animals, if allowed to multiply freely in Petri-dish cultures or on stock slides, after a longer or shorter period of rapid multiplication almost invariably would give evidence of being in the protoplasmic state always observed in conjugating ciliates, aptly described by Calkins ('02) as 'miscible.' In such cultures numerous pairs have been observed, fused by the oral surfaces in a manner very similar to that of normal conjugation. In the first stages this fusion was apparently limited to the peristomial membranelles, although many animals, seemingly with very slight attachment, could nevertheless be fixed and carried through the various processes incident upon staining without being separated. In many cases this fusion was not limited to the pellicle, but showed plainly a definite cytoplasmic connection between the members of a pair (fig. 6), but this connection never became quite so extensive as is usual in normal conjugants of other species of hypotrichs. Occasionally pairs have been observed united by the oral surfaces with the bodies of the two animals somewhat twisted around each other. In figure 5 a pair is shown united by the anterior ends in a manner closely resembling that figured by Maupas ('89, fig. 1, pl. 18) as an initial stage in the conjugation of *Onychodromus grandis*.

From time to time, as these pairs appeared, numbers were isolated for study; in all 100 pairs have been studied. Of these, 3 pairs separated within a few hours after isolation, while the remaining 97 pairs never separated and died in from six to forty-eight hours. Of the animals which separated, one of each pair was stained and the other was transferred to fresh medium on a depression slide. Study of the stained specimens showed a typical condition of the macronucleus in all cases and there was not the slightest sign of any structure which could be interpreted as a definitive micronucleus. The animals which were carried on depression slides continued to divide, thus indicating that whatever may have been the condition of these apparent conjugants, at any rate, it was not pathological.

The appearance and behavior of the animals in cultures, in which pairs were occurring in large numbers, was not different from that of typical, healthy animals. In order to determine definitely if this phenomenon was due to a depressed or pathological state, numbers of single animals were isolated and carried in lines by daily isolations. The division rate of these subcultures indicated that the organisms were in a good physiological condition. Pairs which had been attached for a few hours were separated by forcibly ejecting them from a capillary pipette. Here, again, the division rate did not show that the animals were in a pathological state. It is therefore clear that some other explanation must be sought, both for the onset of the tendency to pair and for the high mortality in permanent pairs.

### 3. *Cytology of Oxytricha hymenostoma*

In view of the fact that the presence of the micronucleus in the hypotrichous ciliates has been insisted upon so strenuously by recent investigators, and since there is an ever-present possibility of overlooking this important structure, every effort has been made in this study to determine its presence. Care has been taken to secure specimens in as many different stages of the life-history as possible, and these have been treated with a large variety of stains, as already mentioned. In addition to total

mounts of single animals and of large numbers at one time by smear preparations, sections have been prepared and studied.

The typical cytoplasmic structure of *Oxytricha hymenostoma* is alveolar throughout, as is so generally found in hypotrichous forms. A greater or less degree of vacuolization occurs at times, particularly when the vitality is low, as shown by the division rate. The macronucleus consists of two more or less ellipsoidal portions connected by a very delicate strand or commissure. This connection may be seen to best advantage in the living animal, as it almost invariably disappears during fixation. The macronuclei (as the two portions of the macronucleus are frequently called) are enclosed in a membrane and consist of chromatinic and achromatinic elements. The latter do not stain, and if the chromatin is overstained, as frequently occurs, are obscured. Proper differentiation, however, will always show these two elements. A 'Kernspalt' is almost invariably seen, most frequently seeming to bisect each portion of the macronucleus, but not uncommonly is found near one end (fig. 8). A typical specimen of *Oxytricha hymenostoma* is shown in figure 4. The cytoplasm here is seen to be somewhat vacuolated while the macronuclei have stained deeply. Both nuclei present a somewhat knobby appearance caused by small projecting portions of chromatin. As this condition has occurred frequently, such macronuclei have been most carefully studied in order to make sure that these projections were macronuclear chromatin. The result has been to establish beyond question of doubt that none of these appearances are micronuclei. A diagrammatically typical macronuclear structure is shown in figure 8.

Throughout the course of the experiments a certain amount of macronuclear fusion has been found, especially in animals in early stages of fission and also at other times. On the other hand, the macronucleus has been observed to be more or less fragmented. This fragmentation has been found to occur more frequently when the division rate was comparatively low. As Woodruff ('05) pointed out, fragmentation of the macronucleus is not necessarily an 'abnormal' condition, since this is a common occurrence during certain stages in the life-history of hypo-

trichous forms and may be succeeded by the entire state, usual at times of high reproductive activity. Essentially the same cytoplasmic and nuclear conditions are revealed by a study of the two pairs shown in figures 5 and 6. In figure 5 the animals are united by the peristomial membranelles, while in figure 6 is shown the typical method of fusion closely resembling an early stage of conjugation. An evident cytoplasmic connection is seen between the two animals.

Pathological specimens, an example of which is shown in figure 7, were obtained when culture A was dying and also in old mass cultures. In these animals both cytoplasm and nuclei stained more lightly than in the typical animal. Except for a slight vacuolization of the macronuclei, there is little difference between the structure of this specimen and of those described previously.

In regard to the question of the presence or absence of micronuclei, a most thorough and long-continued examination of many specimens has been made. As already mentioned, these specimens were prepared during all phases of the life-history of the cultures; during the early period of culture A, when the high division rate indicated that the animal was in good physiological condition; in cultures where there was no doubt that the organisms were in a depressed state, and during the different stages when numerous cases of attempted conjugation occurred. In none of these specimens have any structures been seen which could be interpreted as micronuclei.

#### 4. CANNIBALISM

From the beginning of the experiments on *O. hymenostoma* the appearance of giant specimens in stock and mass cultures was noted. Stained preparations of these giants suggested that such forms had arisen as a result of one animal devouring its sister cells, or, in view of the observations of previous writers, what may be termed cannibalism.

Apparently the earliest account of cannibalism in ciliates was given by Haime ('53) in a short paper on the so-called 'Metamorphosis of *Trichoda lynceus*.' He figured an *Oxytricha* of



'medium size' which contained in the 'digestive cavity' an infusorian of the same species but in 'another stage of development.' Thirty-five years later Maupas ('88) stated that *Onychodromus grandis* under conditions of hunger was cannibalistic, while Joukowsky ('98) described the appearance, in a starved culture, of a large specimen of *Pleurotricha lanceolata* which he believed had attained its great size by eating its own relatives. Later, in cultures of *Onychodromus grandis*, under similar conditions, he saw large specimens, which, upon staining, showed within the cell the nuclear structure of what he interpreted as the remains of ingested animals of the same species.

As the occurrence of giant forms was so universal in mass cultures of *Oxytricha hymenostoma*, and as previous investigators had only referred incidentally to this phenomenon—all evidence submitted to date being largely inferential—it was decided to make a detailed study of this phase of the life-history of *Oxytricha hymenostoma*. In order, if possible, to relate these phenomena to characteristic physiological changes in the life of the cultures, the following study has been made:

1. Observations on the process of swallowing.
2. Digestion of the swallowing animals.
3. A study of the effect of the culture medium on cannibalism.
4. The physiological effect on the animal as shown by the division rate as compared with that of the typical animals.

#### *1. Observations on the process of swallowing*

The actual swallowing of one *Oxytricha* by another was suspected a considerable time before it was actually seen. During the experiment recorded in section 3 many instances were afforded of this act, since it took place on a large scale and under the most favorable conditions for observation, i.e., a large number of animals in the proper state for cannibalism were confined in a relatively small space. Not only has the process been witnessed in the case of cannibals which had already ingested one, two, or more of their fellows, but I have observed frequently the act of one animal swallowing another for the first time.

On January 1, 1918, the following observations were made on a depression-slide culture (2B, see p. 494) in which already many instances of a cannibal swallowing other animals of its species had been seen. One *Oxytricha*, apparently typical, seized a smaller animal by the posterior end (10.55 p.m.). At the end of five minutes its peristomial membranelles could be plainly seen, vibrating in the mouth of the larger animal. At the end of seven minutes (11.02) no sign of the smaller animal was visible. The cannibal was carefully watched for five minutes more, former experience having shown that any attempt to isolate it would probably be followed by egestion of the swallowed animal. The larger animal was then isolated, fixed and stained. Examination of the stained specimen showed the body of the ingested animal somewhat rounded off but still intact, with such structures as the peristomial membranelles and anal cirri distinctly visible. Figure 9 shows a cannibal on which a similar observation had been made, but which had some time previously swallowed another animal. It will be noted in this case that the peristomial membranelles of the ingested animal can still be seen as well as the macronuclei. The posterior macronucleus of the swallowed animal lies just under the anterior end of the posterior nucleus of the cannibal. The remains of the animal swallowed previously are visible as a large faintly staining vacuole in an advanced stage of digestion. Figure 11 shows a cannibal which has just swallowed a fused pair of smaller animals, the macronuclei of which are distinctly visible.

Many cases have been noted of apparently successful attempts to swallow followed by the disgorging of the swallowed animal, which usually seemed none the worse for the experience. Figure 13 shows such a cannibal which had already swallowed five animals and had succeeded in taking in the *Oxytricha* shown in figure 14, but during the operation of isolation the smaller animal was disgorged and swam about actively. A comparison of the size of these animals shows what was invariably found in all observations on swallowing, i.e., the ingested animal, when it is the first animal to be swallowed by a cannibal, is always smaller than the latter. When a culture is in a state favorable

for cannibalism, there has already taken place a rapid increase in the number of organisms in the culture. Consequently, the mean size of such animals has been found to be slightly less than that of the average normal animal. Among these animals which are below average size considerable variation in size occurs and, although the stimulus calling forth the swallowing reaction is present in most of the forms in the culture, the actual swallowing is only successfully accomplished when a relatively large *Oxytricha* attempts to swallow a smaller one.

Cannibalism has been invariably found to occur, throughout the whole course of the study, at the time when numerous pairs are present, attached in the manner so strikingly suggestive of the phenomenon of conjugation. Thus, cannibalism may be interpreted, in a way, as an abortive attempt at conjugation, since it occurs when the organisms are in the physical condition always found in conjugating animals. When the protoplasm of the animals studied in this work is in the 'miscible' condition and individuals happen to unite by the anterior ends, they fuse, and usually remain fused until death occurs. If the anterior end of one animal happens to come in contact with the posterior end of another, swallowing may take place.

It has happened that cannibals have been found whose length was less than that of the average length of the typical animal. The most striking increase is in the breadth of the animal—the effect being comparable to that obtained by the full inflation of an ordinary rubber hot-water bottle. In appearance the cannibal is much darker than the typical single animal, and when several animals have been ingested the dorsal surface often presents a knobby appearance where the pellicle is bulged out by the contained bodies. The pellicle in such a case is stretched almost to the breaking point and considerable care has to be used in handling these cannibals with the capillary pipette to avoid loss of the specimen by bursting.

Table 1 gives a series of measurements made on various cannibals.

It will be noted from a study of the above table that although animals at the inception of the process of cannibalism have a

smaller mean size than usual, there is an increase in both the length and the width of the cannibals depending on the number of organisms ingested. The size of cannibals after the digestion of from two to six bodies is slightly greater than that of the average size of typical animals from cultures.

In the case of the cannibals which underwent fission before digestion of the swallowed animals, the relatively great width was maintained. While the table given above shows a small increase in length and a greater increase in breadth in proportion to the number of bodies contained, great variation has,

TABLE 1

	NUMBER MEASURED	AVERAGE LENGTH IN $\mu$	AVERAGE WIDTH IN $\mu$
Normal Oxytricha.....	30	84.0	38.0
Oxytricha from culture in which cannibals were forming.....	20	60.0	24.0
Cannibals with 1 body.....	20	84.0	43.5
Cannibals with 2 bodies.....	20	86.4	45.4
Cannibals with 3 bodies.....	10	86.5	54.0
Cannibals with 4 bodies.....	4	88.5	55.5
Cannibals with 5 bodies.....	7	96.4	55.5
Cannibals with 6 bodies.....	3	94.0	63.0
Cannibals with 12 bodies.....	1	114.0	66.0
Cannibals with 15 bodies.....	1	135.0	72.0
Cannibals before fission and after digestion of from two to six bodies.....	8	87.3	39.0

nevertheless, been found, e.g., of two cannibals which had swallowed one animal each, one measured  $54 \times 30 \mu$ , the other  $120 \times 51 \mu$ .

One interesting feature in the occurrence of cannibals was the fact that usually when such a form was first seen it had already eaten as many of its fellows as it could hold, while the greatest patience was necessary to witness the process of swallowing the first animal. The rapidity with which cannibals eat considerable numbers of their own species after the initial act of swallowing seems to indicate that the 'swallowing reaction' persists until it is a physical impossibility to ingest any more. Further,

after the first *Oxytricha* has been swallowed, it is a much more simple process to swallow others, since the peristome then is considerably distended (figs. 9, 11, 13, and 16), and accordingly the process goes on rapidly until the cannibal is literally gorged with the bodies of its relatives (fig. 15).

## 2. *Digestion of the ingested animals*

For the purpose of studying the time necessary for the digestion of the ingested animals, thirteen cannibals were isolated from culture 2 B (p. 494) at 8 A.M., December 31st. All had become cannibals since 1 A.M. of the same day, i.e., at approximately the same time. In these cannibals the bodies of the ingested animals could be plainly seen and an accurate count made by using a binocular microscope with a lens combination giving a magnification of about 50 diameters. Of the thirteen animals thus isolated, 3 contained 3 bodies each; 5, 4 bodies each; 2, 5 bodies each; 1, 6 bodies; 1, 7 bodies, and 1 had 8 bodies. Observations were made on these animals for the next fifteen hours and specimens were stained from time to time until a fairly complete series illustrating the whole process of digestion was obtained. A series of measurements was also made during the experiment which shows that the offspring of the cannibals are only slightly above normal size at the end of the second generation.

*Cannibals containing three bodies.* No. 1. At the end of 2 hours the three food vacuoles containing the ingested animals were still distinctly seen. At the end of 3 hours no trace of any of the bodies could be seen, although the animal was still distinctly larger than normal ( $120 \times 51 \mu$ ). It was then stained. The stained preparation showed remains of one body with the macronuclei still present, but stained very faintly.

No. 2. Little change in the appearance of the bodies was noted during the first 2 hours. Gradual disappearance of the outlines of these was noted during the next hour, at the end of which the animal was fixed and stained. The preparation showed the presence of two large vacuoles faintly stained, one



of which contained a chromatin body. The cannibal now measured  $105 \times 54 \mu$ .

No. 3. At the end of 3 hours and 40 minutes little change in appearance of the bodies was noted. At the end of nine hours only one body could be seen, and this had disappeared at the end of 13 hours. The size of the animal now was  $81 \times 42 \mu$ . Study of the stained preparation showed a typical appearance of cytoplasm and macronuclei.

*Cannibals containing four bodies.* No. 4. In 1 hour and 47 minutes the animal had divided by fission with three bodies in the anterior daughter cell,<sup>3</sup> 4a, and one in the posterior, 4p. 4a at the end of 3 hours (from beginning of experiment) showed two bodies distinctly visible. The animal, measuring  $93 \times 54 \mu$ , was then fixed and stained. A glance at figure 16 will show that in the preparation the three food vacuoles are intact, one containing the macronuclei of a swallowed animal, the remaining two staining faintly and containing no chromatin. 4p contained one body which was barely visible at the end of 2 hours. In 3 hours this disappeared completely. The animal was fixed and stained one-half hour later and gave the appearance as of a normal animal except that its size ( $93 \times 54 \mu$ ) was slightly greater than that of the average normal.

No. 5. At the end of 1 hour and 40 minutes four bodies were still distinctly visible. These became much less distinct in  $2\frac{1}{2}$  hours. In  $3\frac{1}{2}$  hours two bodies only could be seen and these were indistinct. At the end of  $4\frac{1}{2}$  hours all bodies were apparently digested. The animal was then stained and had the typical structure of an ordinary vegetative animal, except for two large, faintly staining vacuoles in which no chromatin was visible.

No. 6. In  $1\frac{1}{2}$  hours this animal divided by fission, two bodies passing to each daughter cell. 6a ( $90 \times 57 \mu$ ) was stained at the end of  $2\frac{1}{4}$  hours. Here one body showed the nuclear structure of the ingested animal; the other was smaller, stained faintly, and showed no chromatin. 6p showed presence of both bodies

<sup>3</sup> 'a' denotes that the daughter cell came from the anterior part of the parent and 'p' denotes the daughter cell from the posterior part.

for 5 hours. At the end of 7 hours they could no longer be seen. This animal was kept for 11 hours and 40 minutes, when fission took place. 6pa ( $75 \times 30 \mu$ ) was fixed and stained and was normal in cytological structure (fig. 10). 6pp was stained at the end of  $11\frac{1}{4}$  hours and examination showed that complete digestion of all the ingested animals had taken place. The size of this animal,  $84 \times 36 \mu$ , was practically the same as that of the average noncannibalistic animal.

No. 7. In  $2\frac{1}{2}$  hours the contained bodies could still be distinctly seen. In 2 hours and 57 minutes fission had taken place. 7a, with one body, was kept for 9 hours, at which time the body was still visible. The preparation showed plainly the nuclear structure of the swallowed animal. Size,  $93 \times 45 \mu$ . 7p showed the presence of the three bodies for 9 hours, when they could no longer be seen. The animal was kept for 11 hours, at the end of which it seemed, except for its slightly greater size ( $90 \times 56 \mu$ ), like a typical specimen. The stained preparation made at this time is shown in figure 12. Here the only indication of the ingested animal is the presence of two faintly stained chromatin bodies to the left of the posterior macronucleus of the cannibal. (The specimen is mounted ventral surface up.)

No. 8. At the end of 2 hours only two bodies could be seen distinctly. These gradually disappeared until little sign of any bodies could be seen at the end of 6 hours. In 7 hours fission occurred. 8a was stained and showed details of normal structure. Size,  $75 \times 42 \mu$ . 8p was stained at the end of 14 hours and showed the presence of a vacuole staining faintly and containing no chromatin. Size,  $95 \times 45 \mu$  (fig. 17).

*Cannibals with five bodies.* No. 9. Little change could be observed at the end of 1 hour and 20 minutes. In  $2\frac{1}{4}$  hours only three bodies could be distinctly seen. In 3 hours one body remained visible. The stained specimen showed that this body still preserved its nuclear structure, while remains of two of the other bodies were present as fairly large faintly staining vacuoles containing no chromatin. Size,  $108 \times 51 \mu$ .

No. 10. Bodies remained distinctly visible with little change for 3 hours. These gradually became less distinct until, at the

end of 9 hours, fission took place. 10a, with two bodies still visible in the living animal, showed upon staining that these were in an advanced stage of digestion. 10p. Examination of the living animal showed apparently normal structure. This was verified later by a study of the stained preparation. Size,  $90 \times 45 \mu$ .

*Cannibals with six bodies.* No. 11. Fission occurred in 1 hour and 25 minutes. In each daughter cell two bodies only could be seen. In 12a, at the end of 2 hours and 10 minutes, the bodies were much less distinctly seen. The stained preparation showed that the bodies were nearly digested. Size,  $102 \times 45 \mu$ . 12p was stained at the end of 3 hours. Here digestion had not proceeded so far and the nuclear structure of the swallowed animals could be distinctly seen.

*Cannibals with seven bodies.* No. 12. Little change could be seen at the end of 2 hours and 12 minutes. Fission then began and was completed in 25 minutes. 14a received four bodies and 14p, three. In 12 hours 14a had completely digested the four bodies and was in a prefission stage. 14p. At the end of 9 hours only two bodies could be seen. In 12 hours one body was barely visible. Fission occurred at the end of 13 hours. Half an hour after fission 14pa was stained. Examination of the specimen showed that complete digestion of the ingested animals had taken place (fig. 18). 14pp, stained at the same time, showed a similar result.

*Cannibals with eight bodies.* No. 13 was observed to be in fission 15 minutes after isolation and was stained and mounted. Study of figures 19 and 20 (showing two views of the specimen) reveals the presence of eight bodies, two of which are in an advanced stage of digestion. The anterior daughter cell contains five of these, the remaining three are in the posterior daughter cell. The widths of these daughter cells,  $54$  and  $66 \mu$ , respectively, are considerably greater than that of the average normal animal, although the length of the specimen,  $165 \mu$ , does not indicate that the cells which would have resulted, if fission had been completed would have been longer than an average non-cannibal animal.

All the observations on cannibals have shown that the process of digestion of the eaten animals is a normal one and proceeds rapidly, although there is considerable variation in the time in which equal numbers of swallowed animals are digested by different cannibals. A cannibal usually divides before the bodies of swallowed animals are fully digested. In the second generation complete digestion takes place, as all animals in the third generation which have been examined have been devoid of visible remnants of digestion, although they have a slightly greater size. The result of this experiment shows that the complete history of a cannibal from the first act of swallowing to the almost complete resumption of normal size and appearance may be carried out in time varying from ten to twenty-one hours.

### 3. *The effect of the culture medium*

Four typical animals were isolated on December 20, 1917, from a mass culture coming from stock of culture A. When each had divided twice, four subcultures, 1, 2, 3, and 4, were begun and carried on depression slides, seven drops of medium being used in each case. The animals of subculture 1—composed of four mass cultures, 1A, 1B, 1C, and 1D—were carried in the culture medium and allowed to multiply, the medium not being changed.

The second subculture, composed of four mass cultures—2A, 2B, 2C, and 2D—was carried in the same manner, but the medium was drawn off frequently and fresh medium added.

The animals in the third subculture, composed of four lines—3A, 3B, 3C, and 3D—were kept in the same medium and all the daughter cells but one in each line were removed daily.

The animals in the fourth subculture, composed of four lines—4A, 4B, 4C, and 4D—were carried in the usual way, one animal in each line being transferred to fresh culture daily.

a. *History of the sub-cultures, 1, 2, 3, and 4.* A summary of the history of the first two subcultures is given in tables 2 and 3.

It should be noted that for the first four days there was no essential difference in the division rate of the animals whether

TABLE 2

*History of depression slide mass cultures 1A, 1B, 1C, and 1D of subculture 1. No fresh supply of medium was added. The number of animals observed on each slide is indicated in the columns under the date. Where large numbers were present the closest possible approximation is given. a = animals moving about in the medium more actively than usual. c = a cannibal. pr. att. = two animals fused in a manner similar to that of conjugating animals.*

MASS CULTURE	NUMBER OF ANIMALS											
	Dec. 20	Dec. 21	Dec. 22	Dec. 23	Dec. 24	Dec. 25	Dec. 26	Dec. 27	Dec. 28	Dec. 29	Dec. 30	Dec. 31
1A.....	1	4	16	48±	80± a	60± less a	45 Smaller darker fairly a	45± 4 C	30 n. 3 C	24	32± 2 pr. 1 C	30
1B.....	1	4	16	30	50±	60± very a	65±	60± 2 C	40±	50±	50±	50±
1C.....	1	4	8	50±	30± 2 C	30± 2 C	30±	40± 5 pr. att.	35±	26 5 pr. att.	40±	30±
1D.....	1	4	8	28	50±	50± less a	45±	45±	40± 2 C	45±	40±	38±
Total single non-cannibals.....	4	16	48	156±	210±	200±	185±	190±	145±	145±	162±	148±
Total cannibals.....					2 C	2 C	—	6 C	5 C	—	1 C	—



TABLE 3

*History of depression-slide mass cultures 2A, 2B, 2C, and 2D of subculture 2. The medium here was drawn off daily and fresh medium was added. Other details as in table 2*

MASS CULTURE	NUMBER OF ANIMALS													
	Dec. 20	Dec. 21	Dec. 22	Dec. 23	Dec. 24	Dec. 25	Dec. 26	Dec. 27	Dec. 28	Dec. 29	Dec. 30	Dec. 31	Jan. 1	
2A.....	1	4	16	48±	60±	50± 3 C	80±	80± 2 C	30±	25±	36± 3 C (w. 1 body each)	40± 3 C		
2B.....	1	4	15	45±	60±	100± 3 C	100±	100± 1 C 18 pr. att.	100± 5 C 8 pr. att.	200± 1 C	150± 13 C 40 pr. att.	100± 25 C	70± 22 C None att.	
2C.....	1	4	8	36±	60±	90± 1 C	100± 1 C	90± 12 pr. att.	90± 3 C 10 pr. att.	100± 4 C 20 pr. att.	180± 10 C 2 pr. att.	120± 10 C		
2D.....	1	4	8	24±	40± 1 C contain- ing 1 body	60± 1 C	70	70±	18± (many dead)	12	14 2 C	16		
Total single non-canni- bals.....	4	16	47	163±	220±	300±	350±	340±	238±	347±	380±	276		
Total cannibals.....					10	8	1	3	8	17	28	38		

supplied with fresh culture medium or not. From this time on, however, since no fresh medium was added, both the supply of food became scanty and the excretion products of the animals accumulated, a balanced relation of the animals and their environment was reached, and little or no further multiplication by fission took place. Just at the time this condition was reached, pairs were observed sticking together and cannibals began to appear. In all cases pairs and cannibals were isolated as soon as observed. In the second subculture cannibals appeared on the same day.

It will be seen that the appearance of cannibals in both the first and second subcultures is due essentially to the same cause, since in the latter case the medium was changed daily and the removal of waste products plus the added food supply gave a more rapid increase of the animals. This rapid increase, which in turn tended to bring about a state of balance rapidly by the using up of the food supply and the increase of excretion products, is most marked in mass cultures 2B and 2C. In mass culture 2A an accident on the seventh day led to the loss of over half the animals. The sudden drop in division rate in mass culture 2D I am at a loss to explain. Mass cultures 2B and 2C were most instructive. In these cultures there was an initial rapid multiplication. Just after this period of multiplication had reached its climax, but before a state of equilibrium had been reached, for numerous divisions by fission were still taking place, cannibalism occurred and the animals showed also a strong tendency to become united in pairs. Addition of fresh culture medium prevented a permanent balanced state such as that in the first set of cultures with the result that cannibal formation proceeded steadily until, as in the case of 2B, 20 per cent of the animals on the slide were cannibals.

The division rate for subcultures 3 (see curve on p. 501) and 4 show that in the former set for the first five-day period reproduction took place more rapidly. This seemed somewhat surprising in view of the results obtained by Woodruff ('11) in studying the effect of excretion products on the division rate of *Paramecium*. Evidently in the present study excretion products

did not become a factor influencing the division rate until after the first five days. During the second and third five-day period the division rate of subculture 3 is distinctly lower than that of subculture 4. Here, no doubt, the increasing excretion products had a depressing effect.

In each line of subculture 4 the animals seemed at all times perfectly typical. In mass culture of subculture 3, however, on December 26th, two animals were present on the slide when it was examined. One, as usual, was removed. On the 27th, upon examination it was found that this animal had divided to give two and that these were united peristome to peristome. On the 29th they were more firmly joined and died that night. In this case, since all animals but one had been removed daily it would hardly seem that lack of food had brought on this 'miscible' state, since animals in set 1 were much more numerous on the depression slides and were still dividing, though slowly, but rather that the excretion products of the animals themselves were the underlying factor.

This experiment indicates clearly that cannibalism does not take place while the culture medium is comparatively fresh and also that the greatest amount of cannibalism does not occur in a medium in which the food supply is much depleted, but in one in which the scarcity of food is just beginning to be felt. Thus it seems probable that the accumulation of excretion products plays a part in inciting cannibalism. Jennings ('10) stated that the cause of conjugation was "a decline in the nutritive conditions after a period of exceptional richness that has induced rapid growth and multiplication." These conditions have in this case been duplicated with the result that instead of conjugation cannibalism occurred.

#### *4. The physiological effect of cannibalism*

On December 10, 1917, three apparently typical animals were isolated from three separate stock cultures coming from the original culture A. On December 11th, each animal had divided twice, giving four animals, and these were isolated to form the

four lines of subcultures, 1A, 2A, and 3A. These subcultures were carried with daily isolations until December 26th. On this date two new subcultures, C2 and C3, of four lines each were begun from three cannibals isolated from five-day-old stock cultures of 2A and 3A, respectively. On December 31st two cultures, C1 and CN1, of four lines each were added. The ancestors of these cultures were a cannibal and a non-cannibal, respectively, isolated on the preceding day from a six-day-old stock culture of subculture 1A. As the curves, averaged for five-day periods, showing the division rates of the non-cannibal subcultures (1A, 2A, and 3A) and of the cannibal subcultures (C1, C2, and C3) were essentially identical in each set, they have been combined in figure 2. The curve shows that the division rate of non-cannibal subcultures (1A, 2A, and 3A) was highest during the first three five-day periods and that there was a considerable drop in the division rate, i.e., from 2.4 divisions to 1.4 divisions per day, during the next period, while the average division rate for the cannibal subcultures was considerably higher, i.e., 2.2 divisions. These cannibal subcultures maintained a consistently higher division rate until January 20th to 25th, when it dropped below that of the non-cannibal subcultures for the first time. From this time on till the end of the cultures the division rates are very similar. It should be noted that 3A and 2A died out on February 18th and February 23rd, respectively, whereas subcultures C1, C2, C3, and 3A lived until shortly after March 1st. Subcultures CN1 between January 10th and January 20th had a higher division rate than any of the others, but this rapidly dropped until February 9th, when the subculture died.

The result of this experiment on cannibalism seemed plainly to indicate that animals descended from cannibal ancestors have a higher initial division rate as compared with animals descended from non-cannibal ancestors. This experiment also indicated the possibility, since their life in cultures was somewhat longer, that cannibal progeny are more hardy than progeny of non-cannibal animals.

A second experiment was carried out in a somewhat similar manner. On January 1, 1918, from the three existing lines (3A, 3C, 3D) of subculture 3 (see former history of this subculture on pp 494, 497) were spread out to form three new subcultures, 3A, 3C and 3D, of four lines each and, similarly, from the four lines of subculture 4 (pp. 494, 497) four new subcultures, 4A, 4B, 4C, and 4D, were begun. These subcultures were carried on with daily isolations until January 22nd. The division rates are shown graphically in figure 3.

No cannibalism had occurred in any of the lines of subcultures 3 and 4 of which the subcultures now being considered are lineal

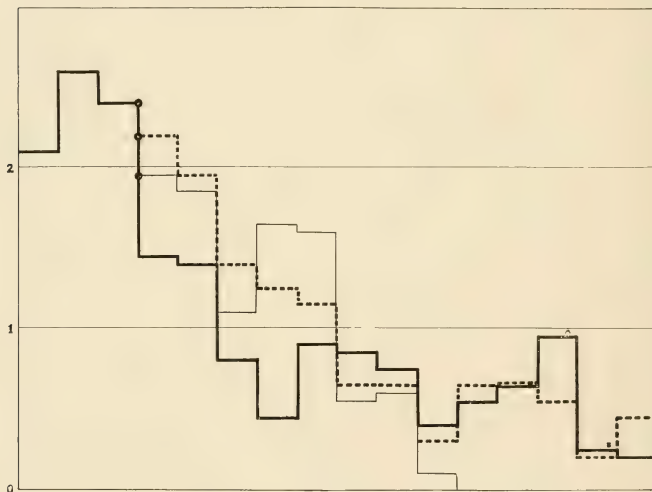


Fig. 2 Comparison of cannibal (broken line) and non-cannibal (continuous heavy and light lines) subcultures. December 12, 1917, to March 1, 1918. Continuous heavy line is a combination curve showing, in five-day periods, the average division rate for non-cannibal subcultures (1 A, 2 A, and 3 A). Dotted line shows a similar curve for cannibal subcultures (C1, C2, and C3) beginning December 26, 1917. Light full line shows the division rate, averaged for five-day periods, for the four lines of the non-cannibal subculture CN1 which died on February 4, 1918. At A the four lines of subculture C3 died (February 19). At B (February 23) the four lines of subculture C2 died. Methods of plotting same as in figure 1.



descendants, and it did not occur on any of the slides of the present subcultures in which daily isolations were made. Cannibalism did occur, however, in stock slides of four or more days standing in each of the twenty-eight lines of these subcultures.

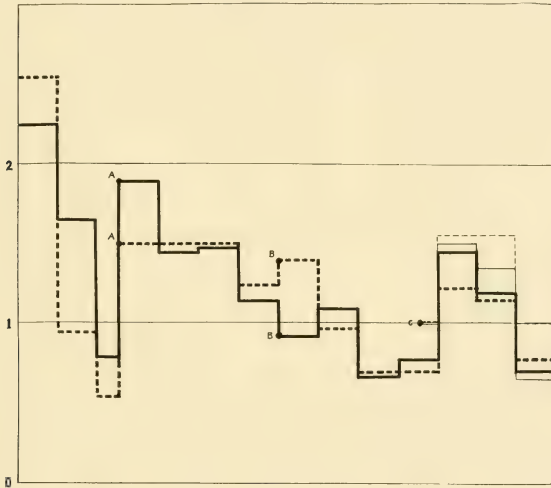


Fig. 3 Comparisons of cannibal (broken line) and non-cannibal (continuous line) subcultures begin at points B and C in this figure, which represents graphically the complete history (December 20, 1917, to February 26, 1918) of subcultures 3 and 4 (section 3) and of all subcultures of the second experiment in this section. The curves in this figure, with the exception of those drawn with light continuous and broken lines, show combined division rates of several subcultures. The broken line from beginning to A (January 2, 1918) represents the division rate of subculture 3; from A to B (January 22, 1918), the averaged division rates of subcultures 3A, 3C, and 3D; from B to the end of the curve, the division rates of the cannibal subcultures 4A-C, 4A-1C, 4B-C, and 3C-C. The continuous line to A shows division rates of subcultures 4; from A to B, the averaged division rates of subcultures 4A, 4B, 4C, and 4D; from B to the end of the curve the same for the non-cannibal subcultures 4A-N, 4A-1N, 4B-N, and 3C-N. The broken line (light) from C (February 9, 1918) to the end of the curve gives the average division rate for subculture 4A-C1, while the continuous line (light) from C to the end of the curve shows the same for subculture 4A-N1. Methods of plotting the same as in figure 1.

For the purpose now of making a comparison of cultures of cannibal and non-cannibal animals, eight new subcultures were begun from these subcultures as follows. On January 22nd from stock slides of four days standing four cannibals were selected—two from subculture 4A to begin subculture 4A-C and 4A-1C; one from subculture 4B to begin subculture 4B-C, and one from subculture 3C to begin subculture 3C-C. In all cases as soon as the animal used to begin the respective subcultures had divided twice, four lines in each subculture were established. Non-cannibal animals, from the same slides from which the cannibals were taken, were selected to form the four lines each of subcultures 4A-N, 4A-1N, 4B-N, and 3C-N. The curve shows that the average division rate for the cannibal cultures (heavy dotted line beginning at B) was higher, 1.40 as compared with .93, than the average division rate for the non-cannibal cultures (continuous line beginning at B) for the first five-day period, but that otherwise little difference can be noted.

On February 9th (continuous and dotted lines beginning at pt. C. in fig. 3) two subcultures, 4A-C1 and 4A-N1, were begun from a cannibal and a non-cannibal, respectively, selected on the previous day from a six-day-old stock culture of 4A-C. The difference in division rate here is not so striking as in the earlier part of the experiment, but the cannibal line is slightly higher during the first full five-day period, and this difference increases in the favor of the cannibal line during the rest of the life of the culture. On February 26th these series of subcultures were discontinued.

The general result of these experiments shows that the effect of cannibalism is to produce an initial higher division rate in the cannibal lines. This higher fission rate may be due merely to the extra nutrition supplied by the ingested animals, in view of the fact that Joukowsky ('98) also found a distinctly higher division rate in cultures of *Pleurotricha* fed on the ciliate, *Uronema*, in hay infusion, than in those kept in infusions of hay, flour, or albumin alone. Since, in this case, however, animals of the same species formed the food in question, there is a possibility, which should not be overlooked, that a more fundamental meta-

bolie change occurs than is implied by mere nutrition, considering that, in the present case, there is an addition of elements derived from similar cytoplasmic and nuclear constituents.

## 5. DISCUSSION

The most recent researches have seemed to show conclusively that, though ciliates may exist in an apparent amiconucleate state at certain periods of their life-history, sooner or later a micronucleus or chromatin equivalent to that contained by a micronucleus takes on a definitive form. Studies on the ciliates, *Opalina*, *Trachelocerca*, *Ichthyophthirius*, and *Blepharisma*, have revealed that their amiconucleate condition exists only during part of the life-history. However, in all the Hypotrichida studied to date a micronucleus has invariably been found as a normal cell constituent, although both temporary or permanent disappearance of this organelle have been described under certain conditions. Thus Maupas ('88) stated that the micronucleus in pathological cultures of *Onychodromus grandis* disappeared entirely. R. Hertwig ('89) questioned this statement and suggested that Maupas might possibly have overlooked the presence of the micronucleus, since he himself had often found great difficulty in observing micronuclei in *Paramecium*.

Calkins ('02) made the positive statement, based on his study of *Paramecium*, that he believed the micronucleus was present at all times in the hypotrichous ciliates. Woodruff ('05) found that in a culture of *Oxytricha* one of the two micronuclei—the posterior—was not present in individuals from the 361st to the 369th generation. On the other hand, Popff ('07), working with *Stylonychia*, found that during depression periods the micronuclei increased in number. Lewin ('11), working with the same form in regeneration, also found an increase in the number of micronuclei. Baitsell ('12) carried a culture of *Stylonychia* for 572 generations and found that micronuclei were present in non-conjugants, conjugants, and ex-conjugants at all stages.

The production of an amiconucleate race of *Paramecium* has been claimed by Lewin ('10), who obtained, by cutting, an amiconucleate fragment which regenerated and produced the 'amiconucleate race.' This race reproduced by fission for nearly two months and was normal in every respect except for the absence of a micronucleus. It is also interesting to note that LeDantec ('97) stated that, in the case of a ciliate (unnamed, with an elongated macronucleus and a single micronucleus, a merozoite containing a portion of the macronucleus only regenerated a micronucleus. The results of both these investigators were published in short preliminary papers and, pending more substantial proof or confirmation by other workers, must be regarded as interesting possibilities rather than as established facts.

The form described in this paper differs from all previously described hypotrichous ciliates in that it has never at any stage in its life-history, since it has been under observation, contained a definitive micronucleus. This statement is made as a result of long-continued and careful cytological study of many preparations of this form throughout the various stages of its life-history during a period of over two years.

As already stated, in other groups than the hypotrichs in which no micronucleus is visible during the vegetative stages it was invariably, during sexual phases of the life-history, found to be present in more or less close connection with the macronucleus, so that the usual dimorphic nucleus was represented apparently during the vegetative condition by a macronucleus alone, i.e., the macronucleus thus was an amphinucleus containing both tropho- and idiochromatin. In this form, therefore, since obvious but abortive attempts to conjugate occurred frequently, there is ground for considering that the nucleus is an amphinucleus, representing both the tropho- and idiochromatic phases.

It is believed by the recent investigators (Woodruff, '05, '13; Baitzell, '14) who have done extensive experimental work on the life-history of the hypotrichida, that these forms will live indefinitely without conjugation or artificial stimulation, pro-

vided an entirely suitable environment be secured. The amicro-nucleate species, *Oxytricha hymenostoma*, has lived continuously under laboratory conditions for over two years. During a considerable portion of this time (July 10, 1917, to April 30, 1918) it has been under continuous observation either on depression slides or in small mass cultures in Petri dishes with the possibility of unobserved conjugation or cannibalism precluded. At the present time (April 30, 1918) the animals in these cultures give every evidence of being able to live indefinitely. The conclusion, therefore, in this case again, is that the only requisites for the continued existence of this form are favorable environmental conditions.

The same conclusion is reached from experiments in which every effort was made to induce conjugation. Although no true process of conjugation has been obtained, there is every reason to believe that the organisms have frequently been in a general physiological condition similar to that of conjugating animals. The entire absence of any of the usual nuclear phenomena attendant upon conjugation has confirmed the belief that this form not only possesses no micronucleus, but also is apparently lacking in the chromatinic material necessary for carrying out the process of syngamy. The continued existence of such an organism indicates, therefore, that conjugation though usually taking place in all other hypotrichous forms, may be entirely dispensed with without loss of viability.

In cases where syngamy has not been observed there are three abstract possibilities (Minchin, '12): first, that it occurs, but has not been seen; secondly, that it is in abeyance; thirdly, that it is primarily absent, i.e., has never occurred in the life-history of the form. The fact that syngamy is of such general occurrence in the protozoa renders the first of these possibilities the most probable. In this case, however, there is every reason to believe that syngamy has been repeatedly attempted, but has never been carried out for the obvious reason that this form lacks the nuclear constitution necessary for the process. The fact that periodic attempts to conjugate occur indicates the probability that it has occurred in the past history of this organ-



ism. It is probable that this form at some time in its history contained a micronucleus, though it is of little value to speculate concerning the manner in which the present amiconucleate condition arose, since there are no data at hand. Such a condition might conceivably arise by some abnormal process during conjugation which resulted in suppression of differentiation between macro- and micronuclear material.

Although the significance of the process of conjugation has been exhaustively studied since Maupas' time, a complete solution of the problem has not as yet been obtained. The existence of a form which not only apparently may live indefinitely without conjugation, autogamy, or endomixis (assuming the possibility of the latter phenomenon in an hypotrichous form), but also apparently does not possess the ability to undergo any of these phenomena, brings to light an entirely new possibility in the life-history of ciliates. It has been proved quite conclusively (Woodruff, '14) that in forms which ordinarily conjugate, the continued prevention of this process brings about no loss of viability if a favorable environment be provided. However, in the organism under consideration there is apparently no possibility not only of conjugation or endomixis, but also of autogamy, and thus we have from another source crucial evidence that none of these phenomena is an indispensable factor in the life-history of this hypotrichous form.

#### 6. SUMMARY

1. A pedigreed culture of *Oxytricha hymenostoma* has been carried for 289 generations, from July 10, 1917, to November 17, 1917, and since that time to date (April 30, 1918) by means of small mass cultures in petri dishes.

2. A micronucleus has not been seen at any time in any of the animals during the history of the cultures.

3. Syngamy has not occurred during the course of the experiments, although it is believed that the animal has frequently been in a physical state similar to that in which syngamy takes place.

4. While in this state, *a*) animals fuse in pairs in a manner similar to that of conjugating animals; *b*) cannibalism takes place.

5. Animals, fused as described in 4a, either remain fused until death occurs or separate. In the latter case the organisms continue to reproduce and give no signs of being in a depressed condition.

6. When cannibalism has occurred, digestion of the ingested animals proceeds rapidly and a return to the typical size and structure soon takes place.

7. Cannibalism has the effect of raising the division rate somewhat for a short time.

8. This amiconucleate race of *Oxytricha hymenostoma* apparently can live indefinitely under favorable environmental conditions without conjugation, autogamy, or endomixis. Indeed its amiconucleate condition seems to preclude the possibility of the occurrence of these phenomena.

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## PLATES

All of the figures in plates 1 and 2 are microphotographs of permanent preparations (total mounts) stained with Delafield's hematoxylin and in most cases counterstained with tetra-brom-fluoresceic acid. The use of other counterstains is denoted in the explanation of figures. The same magnification (525 diameters) was used in all cases with the exception of figures 8, 15, 18, and 19, which are magnified 375 diameters.

## PLATE 1

### EXPLANATION OF FIGURES

4 A typical normal individual from culture A in the fiftieth generation. July 24, 1917 (dorsal view).

5 Typical pair from stock of culture A fused in the peristomial regions.

6 Typical pair from stock of culture A. Appearance closely simulating that of conjugating animals (Del. hem.).

7 Pathological specimen from stock of culture A in 283rd generation, November 6, 1917. Preparation made at the end of the fourteenth day without division.

8 A typical cannibal with remains of five ingested animals, two of which are in an advanced stage of digestion. A kernspalt is present in each macronucleus of the cannibal (dorsal view).

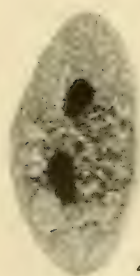
9 A cannibal which has just ingested an animal, the peristome of which is visible. The posterior macronucleus of the ingested animal is partially obscured by the macronucleus of the larger animal. A large vacuole containing the remains of an individual now almost completely digested is at the left of the posterior macronucleus of the cannibal (ventral view).

10 An individual, descendant of a cannibal in the second generation (No. 6, p. 491).

11 A cannibal which has just ingested a pair of animals which were fused as if conjugating. The peristome is much distended.

12 An individual, descendant of a cannibal in the first generation, which shows almost complete digestion of ingested animals. The remains of the macronuclei of an ingested animal are visible as two chromatin bodies on the left of the posterior macronucleus (ventral view) (p. 492).





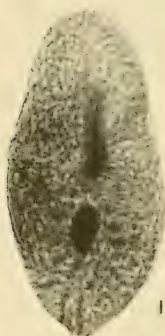
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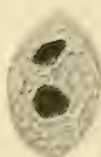
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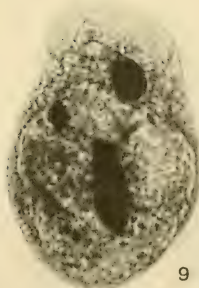
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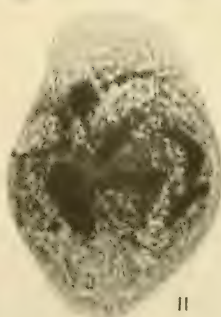
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## PLATE 2

### EXPLANATION OF FIGURES

13 A cannibal which has ingested five animals and disgorged the individual shown in figure 14.

14 Individual considerably under normal size which was swallowed and shortly after disgorged by animal shown in figure 13.

15 A cannibal in early fission stage containing the bodies of sixteen ingested animals, thirteen of which show in the figure (dorsal view) (Del. hem. only).

16 An individual, descendant of a cannibal in the first generation, showing the presence of the bodies of three ingested animals. The vacuole to the right of the fused macronucleus of the cannibal shows the macronuclei of one ingested animal (dorsal view) (p. 491).

17 An individual, descendant of a cannibal in the first generation, showing one body (to the right of the posterior macronucleus of the cannibal) in an advanced stage of digestion (dorsal view) (p. 492).

18 An individual, descendant of a cannibal, showing complete digestion of ingested animals (compare with fig. 4).

19 and 20 Dorsal and lateral views of a cannibal in fission containing the bodies of eight ingested animals (no. 13, p. 493).



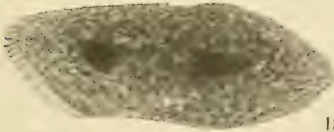
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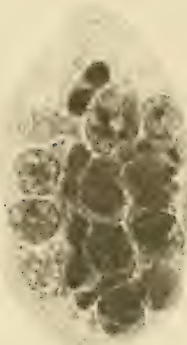
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14



17



15



16



## SUBJECT AND AUTHOR INDEX

<b>A</b>	CIDS to ciliate Infusoria. The toxicity of.....	443
	Albino rat. Studies on inbreeding. IV. A further study of the effects of inbreeding on the growth and variability in the body weight of the.....	71
	(Albino rat). Studies on the reputed endocrine function of the thymus gland.....	311
	Amicronucleate Oxytricha. I. Study of the normal animal, with an account of cannibalism. An experimental study of an.....	473
	Amphibia as affected by thyroidectomy. Growth and development of.....	1
	Apis mellifera L. The photic reactions of the honey-bee.....	343
	Arcella dentata and A. polypora. The effects of environmental factors upon the heritable characteristics of.....	427
	AREY, LESLIE B., AND CROZIER, W. J. The sensory responses of Chiton.....	157
	AREY, LESLIE B., CROZIER, W. J., AND. Sensory reactions of Chromodoris zebra.....	261
<b>B</b>	ODY weight of the albino rat. Studies on inbreeding. IV. A further study of the effects of inbreeding on the growth and variability in the.....	71
<b>C</b>	ALKINS, GARY N. Uroleptus mobilis Engelm. II. Renewal of vitality through conjugation.....	121
	Cannibalism. An experimental study of an amicronucleate Oxytricha. I. Study of the normal animal, with an account of.....	473
	Characteristics of Arcella dentata and A. polypora. The effects of environmental factors upon the heritable.....	427
	Chiton. The sensory responses of.....	157
	Chromodoris zebra. Sensory reactions of.....	261
	Ciliate Infusoria. The toxicity of acids to.....	443
	COLLETT, M. E. The toxicity of acids to ciliate Infusoria.....	443
	Conjugation. Uroleptus mobilis Engelm. II. Renewal of vitality through.....	121
	Crepidula plana. III. Transference of the male-producing stimulus through sea-water. Studies on sex in the hermaphrodite mollusc.....	113
	CROZIER, W. J., AND AREY, LESLIE B. Sensory reactions of Chromodoris zebra.....	261
	CROZIER, W. J., AREY, LESLIE B., AND. The sensory responses of Chiton.....	157
<b>D</b>	AWSON, J. A. An experimental study of an amicronucleate Oxytricha. I. Study of the normal animal, with an account of cannibalism.....	473
	Development of Amphibia as affected by thyroidectomy. Growth and.....	1
<b>E</b>	NDOCRINE function of the thymus gland (albino rat). Studies on the reputed.....	311
	Environmental factors upon the heritable characteristics of Arcella dentata and A. polypora. The effects of.....	427

<b>F</b>	ACTORS upon the heritable characteristics of Arcella dentata and A. polypora. The effects of environmental.....	427
	Function of the thymus gland (albino rat). Studies on the reputed endocrine.....	311
<b>G</b>	LAND (albino rat). Studies on the reputed endocrine function of the thymus.....	311
	GOULD, HARLEY N. Studies on sex in the hermaphrodite mollusc Crepidula plana. III. Transference of the male-producing stimulus through sea-water.....	113
	Growth and development of Amphibia as affected by thyroidectomy.....	1
	Growth and variability in the body weight of the albino rat. Studies on inbreeding. IV. A further study of the effects of inbreeding on the.....	71
<b>H</b>	EGNER, ROBERT W. The effects of environmental factors upon the heritable characteristics of Arcella dentata and A. polypora.....	427
	Heritable characteristics of Arcella dentata and A. polypora. The effects of environmental factors upon the.....	427
	Hermaphrodite mollusc Crepidula plana. III. Transference of the male-producing stimulus through sea-water. Studies on sex in the.....	113
	Honey-bee, Apis mellifera L. The photic reactions of the.....	343
	HOSKINS, E. R., AND HOSKINS, M. M. Growth and development of Amphibia as affected by thyroidectomy.....	1
	HOSKINS, M. M., HOSKINS, E. R., AND. Growth and development of Amphibia as affected by thyroidectomy.....	1
<b>I</b>	NBREEDING. IV. A further study of the effects of inbreeding on the growth and variability in the body weight of the albino rat. Studies on.....	71
	Infusoria. The toxicity of acids to ciliate.....	443
<b>K</b>	ING, HELEN DEAN. Studies on inbreeding. IV. A further study of the effects of inbreeding on the growth and variability in the body weight of the albino rat.....	71
<b>M</b>	ALE-producing stimulus through sea-water. Studies on sex in the hermaphrodite mollusc Crepidula plana. III. Transference of the.....	113
	MINNICH, DWIGHT E. The photic reactions of the honey-bee, Apis mellifera L.....	343
	Mollusc Crepidula plana. III. Transference of the male-producing stimulus through sea-water. Studies on sex in the hermaphrodite.....	113
<b>O</b>	XYTRICHA. I. Study of the normal animal, with an account of cannibalism. An experimental study of an amicronucleate.....	473



- P**HOTIC reactions of the honey-bee, *Apis mellifera* L. The..... 343
- R**AT. Studies on inbreeding. IV. A further study of the effects of inbreeding on the growth and variability in the body weight of the albino..... 71
- Rat). Studies on the reputed endocrine function of the thymus gland (albino)..... 311
- Reactions of *Chromodoris zebra*. Sensory... 261
- Reactions of the honey-bee, *Apis mellifera* L. The photic..... 343
- Renewal of vitality through conjugation. *Uroleptus mobilis* Engelm. II..... 121
- Responses of *Chiton*. The sensory..... 157
- S**ENSORY reactions of *Chromodoris zebra* 261
- Sensory responses of *Chiton*. The..... 157
- Sex in the hermaphrodite mollusc *Crepidula plana*. III. Transference of the male-producing stimulus through sea-water. Studies on..... 113
- Stimulus through sea-water. Studies on sex in the hermaphrodite mollusc *Crepidula plana*. III. Transference of the male-producing..... 113
- T**AKENOUCHI, MATSUZIRO. Studies on the reputed endocrine function of the thymus gland (albino rat)..... 311
- Thymus gland (albino rat). Studies on the reputed endocrine function of the..... 311
- Thyroidectomy. Growth and development of *Amphibia* as affected by..... 1
- Toxicity of acids to ciliate *Infusoria*. The... 443
- U**ROLEPTUS *mobilis* Engelm. II. Renewal of vitality through conjugation.. 121
- V**ARIABILITY in the body weight of the albino rat. Studies on inbreeding. IV. A further study of the effects of inbreeding on the growth and..... 71
- Vitality through conjugation. *Uroleptus mobilis* Engelm. II. Renewal of ..... 121
- W**EIGHT of the albino rat. Studies on inbreeding. IV. A further study of the effects of inbreeding on the growth and variability in the body..... 71







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